TEMPORAL CATHEPSIN AND MATRIX METALLOPROTEINASE ACTIVITY IN TENDON EXTRACELLULAR MATRIX DAMAGE

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TEMPORAL CATHEPSIN AND MATRIX METALLOPROTEINASE ACTIVITY IN TENDON EXTRACELLULAR MATRIX DAMAGE

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To my loving family and friends, and the Knowles-Carter family

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LIST OF SYMBOLS AND ABBREVIATIONS

$ApoE^{-/-}$	apolipoprotein E deficient
cat	cathepsin
$catK^{-/-}$	cathepsin K deficient
CT_X	c-terminal telopeptide
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPIC-μCT	equilibrium partitioning of ionic contrast agent via micro-computed tomography
ER	endoplasmic reticulum
GAG	glycosaminoglycan
H&E	hematoxylin and eosin
ICTP	carboxyterminal telopeptide of type I collagen
MMP	matrix metalloproteinase
μCT	micro-computed tomography
NLO	non-linear optics
NT_X	n-terminal telopeptide
OCT	optimum cutting temperature
OA	osteoarthritis
PBS	phosphate-buffered saline
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHG	second harmonic generation
WT	wild type

SUMMARY

Tendinopathy, or tendon overuse, is one of the most common musculoskeletal disorders affecting athletes, laborers, and aging adults. If left untreated, overuse injury can progress to partial or full-thickness tears that require surgical intervention. In order to develop preventative treatment strategies, it is important to identify the factors that initiate tendon damage. The multifactorial etiology of tendon overuse includes imbalances in proteases and their endogenous inhibitors that lead to the degradation of extracellular matrix (ECM). Cysteine cathepsins and matrix metalloproteinases (MMPs) have been implicated in tendon pathogenesis. Though several studies have published the effects of a single protease on tendon degradation, this does not provide a complete picture of a very dynamic proteolytic network at play in a pathologic environment. Cells can produce and secrete many proteases and endogenous inhibitors simultaneously that can interact with each other and impact matrix degeneration. It is not well understood how cathepsins and MMPs work cooperatively within and between families to effectively degrade tendon ECM. Our lab has identified a temporal regulation of cathepsin K and cathepsin L in a rat model of rotator cuff overuse, demonstrating upregulation of cathepsins K and L early in the injury time course, while only cathepsin L continued to be upregulated during later time points.

The objective of this research is to investigate the proteolytic contribution to tendon damage. It is hypothesized that cathepsins, especially cathepsin K as the most potent collagenase, act in sequence to facilitate ECM degeneration. This study uses a rat model of rotator cuff tendinopathy to determine the temporal roles of cathepsins and MMPs in supraspinatus tendon overuse injury, as well as a mouse Achilles tendon model

to determine the sequential actions of multiple cathepsins on tendon matrix degradation. Together, this work determines the biochemical roles of cathepsins and MMPs on tendon damage and elucidates mechanisms of protease regulation. Understanding how the dynamic proteolytic network contributes to tendon degradation will better inform clinical treatment strategies to prevent progression to tendon failure.

CHAPTER 1. INTRODUCTION

Tendinopathy is one of the most common musculoskeletal disorders diagnosed in athletes and laborers, and is generally attributed to overuse. Though several intrinsic and extrinsic etiological factors have been implicated in tendinopathy, the cascade of events that leads to tendon failure is still not well understood. A clinical hallmark of tendinopathy is degeneration of extracellular matrix (ECM) components, specifically type I collagen. It has been shown that matrix metalloproteinases (MMPs), and more recently, cathepsins—a family of cysteine proteases including the most powerful human collagenases and elastases—are upregulated in tendinopathic tissues after overuse. However, the cooperative effect of proteases on collagen degradation in tendon ECM still needs to be investigated. Limited knowledge regarding tendon pathology progression has hindered the development of preventative methods and treatment options in early stage tendinopathy.

1.1 Research Objectives and Specific Aims

Our long-term goal is to identify key markers of ECM remodeling in tendon overuse and exploit their mechanisms of action to help develop potential therapeutic strategies. The **overall objective of the dissertation** is to investigate the proteolytic contribution to tendon damage using rodent models and type I collagen gel matrix. The **central hypothesis** is that cathepsins play a sequential role in ECM degeneration and progression of tendinopathy. The rationale is that understanding how multiple proteases contribute to tendon degeneration will better inform clinical treatment strategies and future potential regenerative therapies to prevent tendon failure. The central hypothesis will be tested with these specific aims:

Aim 1: Determine the temporal roles of proteases in rat supraspinatus tendon overuse injury. *Hypothesis: Cathepsin upregulation in the supraspinatus tendon insertion tissues will peak at the earliest point of injury in a rat model of rotator cuff overuse. It is also hypothesized that both supraspinatus tendon insertion and nearby articular cartilage tissues will show evidence of damage and proteolytic upregulation at 10 weeks of overuse.* Skeletally mature Dahl Salt-Resistant rats were obtained and subjected to a downhill treadmill model of rotator cuff overuse injury as described previously (n=6/group/time point) for 2 or 10 weeks and compared to age-matched controls. At each time point, the insertion region of supraspinatus tendons and articular cartilage was harvested from control and overuse animals for histological and biochemical analysis. Supraspinatus tendons subjected to overuse for 10 weeks exhibited significant evidence of damage as quantified by histological scoring. Additionally, injured animals after 10 weeks demonstrated significant decrease in cartilage thickness and increased cartilage attenuations, suggesting decreased glycosaminoglycan content. However, no upregulation of cathepsins or MMPs was quantified at either the 2 or 10 week time points in supraspinatus tendon or articular cartilage. This suggested that peak proteolytic upregulation occurred between the time points, as published previously by our lab. These findings are discussed in chapter 3.

Aim 2: Determine the cooperative and interactive effects of cathepsins on collagen and tendon matrix degradation. *Hypothesis: Cathepsin K, the most potent mammalian collagenase, initiates collagen degradation by exposing sites further susceptible to hydrolysis by mouse cathepsin L.* Our rat model of rat rotator cuff tendinopathy in

previous work demonstrated an upregulation of cathepsin K and cathepsin L at 4 weeks of injury, with only cathepsin L continuing to be upregulated after 8 weeks with evidence of tendon damage by the end of the time course. This suggested that the sequential point of upregulation for each cathepsin was necessitous to the eventual tissue degeneration. We developed collagen gel and mouse tendon ECM models to use as test beds to investigate the role of sequential co-incubations of cathepsin K and cathepsin L on matrix degradation and enzyme activity. We found increased degradation of matrix primed, or pre-incubated, with cathepsin K before co-incubating with cathepsin L when compared to matrix that was concurrently co-incubated with cathepsins K and L. These cathepsins also exhibited substrate-dependent bi-directional cannibalism not shown in enzyme co-incubations without substrate. Furthermore, tendon ECM protected active cathepsins K and L from autodigestion and sustained activity over time. These data suggested that addressing the sequential actions of cathepsins K and L on tendon matrix may be critical for effective inhibition strategies. These findings are discussed in chapter 4.

1.2 Significance and Innovation

The complex proteolytic network contributing to tendon degradation is not very well understood. Cathepsins and MMPs have separately been implicated in tendon pathogenesis, but studies are still needed to investigate how cathepsins and MMPs and their inhibitors interact within and between these two families. The **overall objective** of this research is to investigate the proteolytic contribution to tendon damage. We have recently discovered a temporally dependent upregulation of cathepsin activity in early stage tendinopathy after 4 and 8 weeks of overuse. However, in order to understand how this regulation is biochemically relevant in the context of proteolytic actions of multiple

proteases, more studies are necessary. Here, we use both *in vivo* and *in vitro* models of tendon degradation to understand proteolytic mechanisms of ECM degeneration. The **central hypothesis** is that cathepsins and MMPs play a sequential role in ECM degeneration and disease progression.

This research is significant because it aims to gain fundamental understanding of the temporal proteolytic regulation between two different proteolytic families and how this regulation is biochemically relevant for tendon ECM degradation. The rationale is that understanding how these two families of enzymes contribute to tendon degeneration will better inform clinical inhibition-based treatment strategies and future potential regenerative therapies to prevent full tendon tears.

Although studies have shown that MMPs are involved in tendinopathic development [1–3], as well as upregulated in end stage tendon disorders [2,4], further studies are needed to investigate the proteolytic profile of both cathepsins and MMPs in tendinopathy. Adding to the data published by this lab, by multiplex gelatin zymography, cathepsins and MMP profiles are evaluated at broader time points in the same model of rat supraspinatus overuse used previously [5]. This provides information about how these proteases are regulated over time in an injury context in a manner not demonstrated in other studies.

Additionally, this research employs two models of matrix degradation that incorporate multiple cathepsins incubated in varied sequence and measures the amount of matrix degradation and changes in the amount of present cathepsins over time after co-incubation. Few other studies have examined the biochemical mechanisms of how these enzymes synergistically work to degrade insoluble collagen. In total, this work is

innovative because it provides mechanistic data on sequential proteolytic degradation of tendon that is key to understanding and preventing tendon pathology.

CHAPTER 2. LITERATURE REVIEW

2.1 Cysteine Cathepsins and Extracellular Matrix Degradation

Proteases are enzymes that catalyze the irreversible hydrolysis reaction on peptide bonds. There are more than 500 human proteases, and their proteolytic actions are crucial for many homeostatic functions such as immune function and antigen presentation, digestion, cell cycle, apoptosis, coagulation, and degradation of other cellular proteins [6–8]. They also are involved in many pathological conditions such as cardiovascular disease, cancer, osteoporosis, arthritis, and inflammation [6,9,10]. The five major types of proteases, as defined by the mechanism of hydrolysis at the active site are serine, metallo, aspartic, threonine, and cysteine proteases [6].

2.1.1 Cysteine Cathepsins

2.1.1.1 Cysteine Cathepsin Classification and Localization

Novel insights from the last 50 years have seen cathepsins emerge as major players in cellular regulation, protein turnover, and disease progression [6,7,11–13]. Cathepsins have various classifications including serine cathepsins (A and G), aspartic cathepsins (D and E), and cysteine cathepsins [11,13,14]. Cysteine cathepsins are the largest group of proteases that belong to the papain superfamily in the clan CA, C1a family of cysteine proteases [6,8,14]. 11 human cysteine cathepsins have been identified by genomic sequencing (cathepsins B, C, F, H, K, L, O, S, V, X, and W) [14].

Cysteine cathepsins are highly homologous proteases, but particularly, cathepsins K, L, S, and V share 60% sequence identity but maintain unique cellular functions [6,8,15–17]. Cathepsins B and L are ubiquitously expressed in lysosomes of most human

cells [16], while others are not. Cathepsin V has been found in the thymus, testis, brain, corneal epithelium, and skin, and cathepsin S is expressed predominantly in antigen presenting immune cells [14,16]. Cathepsin K was first identified in osteoclasts and ovaries [6,11,12,16,18] but has also been found in synovial fibroblasts and rheumatoid arthritic joints [7,9,11,14,17]. Upregulated catK levels were also found in human calcific tendons and a rabbit model of flexor tendon injury [19,20]. Genomic sequencing has identified all of the cathepsins encoded for humans and mice, however not all human cathepsins have homologous mouse equivalents. Cathepsin K is known as the most potent mammalian collagenase [21] and is the only cysteine cathepsins that is unambiguously implicated in bone resorption in both humans and mice [22]. Interestingly, human cathepsin L, human cathepsin V, and mouse cathepsin L while all very homologous, sharing 75% sequence identity, all have differing expression and functions [8]. Eight evolutionarily related cathepsin sequences were found to be expressed exclusively in mouse placenta [8]. Human cathepsin V, not human cathepsin L, is the ortholog to mouse cathepsin L [8,15,23], and thus it is imperative to consider their functional differences when relating human and mouse pathologies. Human cathepsin V is the most powerful mammalian elastase and has been implicated in cancer and autoimmune disorders [8,24,25]. Mouse cathepsin L is implicated in diabetes and cardiovascular disease [24,26].

Cysteine cathepsins were first found in lysosomes, part of the approximately 50 known lysosomal enzymes. Because of this, cathepsins were thought to be exclusively intracellular proteases with general, non-selective proteolytic specificity in the lysosomal compartments where they degrade both intracellular and extracellular substrates

[7,11,12]. This view dominated the field two decades ago, especially because cathepsins are optimally active in an acidic and reducing environment, with the exception of cathepsin S which maintains stability and activity at neutral or slightly basic pH [11,12,17]. However, cathepsins have since been found outside of the lysosome in cytosolic and pericellular spaces as well as associated with the cell membrane, expanding the limited view of cathepsin behavior and honing in on their functional specificity [14]. For example, cathepsins have been observed in the cytosol during apoptosis [27,28], and cathepsins B and L have been found associated with the cell membrane of colorectal carcinoma and melanoma cells [14,29,30]. Additionally, cathepsins are secreted in extracellular spaces in normal physiological function as with cathepsin K secretion by osteoclasts to facilitate bone resorption, but also in pathological conditions, such as the synovium of arthritic patients [9,14].

2.1.1.2 Regulation of Cathepsin Activity

Cathepsins are powerful enzymes with broad substrate specificity [11] and due to this proteolytic potential in the lysosome and in the extracellular space, they are tightly regulated [6,14]. Cathepsins are translated as inactive zymogens in the endoplasmic reticulum (ER) with an additional prepropeptide sequence on the N-terminal end that blocks the active site. Interestingly, most of the differences between these highly homologous enzymes are found in the propeptide sequence. This presents as a way to selectively inhibit specific cathepsins when many are present in the same subcellular compartment [7]. The prepropeptide sequence serves as a signaling molecule that traffics cathepsins through the rough ER to the Golgi, and during this transport the pre-region is cleaved. In the Golgi, procathepsins typically undergo post-translational glycosylation

tagging with mannose-6-phosphate, as the propeptide acts as an inhibitor to prevent unintended autodigestion, or catalysis of a cathepsin by itself [6,11]. This glycosylation then targets cathepsins to endolysosomal or lysosomal compartments where the enzyme will be activated, or trafficked to the membrane for secretion into the pericellular space. This propeptide sequence must be cleaved in order to become a mature and active enzyme [8,14,16]. The propeptide can be autocatalytically cleaved under acidic conditions, where a cathepsin molecule cleaves its own propeptide sequence, or the cathepsin can be activated by other mature cathepsins [11].

Additional endogenous regulators of cathepsin activity are the multiple classes of cathepsin inhibitors. The largest class of inhibitors is the cystatin superfamily. Cystatins are competitive, reversible, tightly binding inhibitors that prevent substrates from being cleaved by competitively blocking their entrance into cathepsin active sites. This family includes the types 1, 2, and 3 cystatins. Type 1 cystatins, or stefins (cystatins A and B), are only intracellular inhibitors. Type 2 cystatins include cystatin C, which is the most abundant extracellular cysteine protease inhibitor. Type 3 cystatins, also known as kininogens, are multifunctional, multidomain glycoproteins that are predominantly found in blood plasma. Other endogenous inhibitors include serpins that inhibit serine proteases, but can also inhibit cathepsins K, L, and S, and herpins that specifically inhibit cathepsin L [6,11,13,14,31]. Given that cathepsins have many potential substrates, restriction to lysosomal compartments contains active cathepsins, and prevents the degradation of unintended substrates. However, when cathepsins escape these sequestering compartments or are trafficked to the cellular membrane for secretion, under homeostatic and pathological conditions, the presence of endogenous inhibitors keep the

proteolytic actions of cysteine cathepsins in check [16]. The dysregulation of cathepsins and inhibitor levels are what lead to breakdown of extracellular matrix (ECM) proteins [1,2,14,32].

2.1.2 Extracellular Matrix Degradation

The extracellular matrix is heterogeneous and tissue-specific. ECM fills the interstitial space between cells or clusters of cells, and makes up basement membrane that provides support for resident cells [33]. It is fundamentally composed of structural proteins, proteoglycans and water [14,33], with fibrous proteins and proteoglycans being the main classes of macromolecules. Normal remodeling of the ECM occurs in homeostatic conditions, but this remodeling can lead to degeneration of the ECM with imbalance of proteases and their inhibitors [14]. Many fibrous protein and proteoglycan macromolecules are established substrates of cathepsins [11,14,21,30,34,35]. Cathepsins can degrade these extracellular matrix components extracellularly as well as intracellularly in the lysosomal compartments when matrix proteins are endocytosed by cells. [14].

Collagen is the most abundant protein present in mammals, makes up about 30% of total protein mass, and serves as the main structural protein of the extracellular matrix in many tissues [14]. There are 20 members of the collagen superfamily. A common characteristic of collagen is the triple helical structure of the molecules consisting of three polypeptide α chains, the amount of which varies between types of collagen. Collagen fibrils display a D-periodicity banding pattern of about 67nm and self-assemble into staggered microfibrils ranging from 15 to 500nm in diameter, depending on the tissue. These fibrils continue to self-assemble with nearby microfibrils in a hierarchical manner

to form a tissue unit [36]. Many tissue destructive diseases are characterized by the loss of fibrillar collagens, specifically types I and II collagen. Fibrillar collagens can be heterotypic, comprised of several collagen types in various tissues [36].

Type I collagen represents the vast majority of structural collagens [14]. The type I collagen molecule is triple helical and consists of two α1(I) and one α2(I) chains that follow repeating Gly-Pro-X, or with hydroxyproline (Hyp), Gly-X-Hyp amino acid sequences [14,37–39] (Fig 2.1). A distinguishing feature of cathepsin K is that it is the most potent mammalian collagenase, able to cleave at the telopeptide sequences in addition to multiple sites within the collagen molecule that other collagenases are not able to cleave. Part of this is attributed to the fact that cathepsin K can accept proline residues near the scissile region of its active site [14,21]. Although not as potent, cathepsins L, V, and S have collagenolytic potential as well [14,21]. Elastin is another major fibrillar protein that makes up the ECM. Elastin fibers provide elastic stretch and recoiling properties for tissues that experience repeated stretching forces [14,33]. While cathepsin V is the most potent mammalian elastase, cathepsins K, L, and S are also able to degrade elastin [8,11,23,24].

Collagen fibrils and elastin fibrils also form heterogeneous macromolecules with proteoglycans that influence collagen and elastin crosslinking, tissue properties, and impact proteolytic potential of cathepsins. Proteoglycans are the most abundant component of the interstitial matrix. They are protein-polysaccharide complexes with a protein core covalently bound to glycosaminoglycan (GAG) molecules, which are polysaccharide repeats. These molecules are highly hydrophilic, drawing water into the

ECM, allowing fibers to slide past one another, and providing viscoelastic properties [40–42]. The types of GAGs present vary by tissue and possess many unique properties.

GAGs can have substantial impacts on cysteine cathepsins; rate of autocatalytic activation of cathepsins can be accelerated by the presence of GAGs [11], and collagenolytic activity of cysteine cathepsins can also be mediated by GAGs. Chondroitin sulfate can enhance the collagenolytic activity of cathepsin K, while dermatan, heparan sulfate, and heparin can reduce cathepsin activity [25]. Chondroitin sulfate also inhibits the elastinolytic activity of cathepsins K and V, showing varied influence on cathepsin activity between substrate types [14].

2.2 Tendinopathy

Tendinopathy is an umbrella term that encompasses many stages of disease progression. Tendon injuries may occur due to acute trauma that is coupled with a significant inflammatory response or injuries may occur due to chronic degeneration that can lead to partial or full-thickness tendon tears [2,43]. The etiology of tendon rupture is unclear, but most commonly stems from chronic tendinopathic degeneration [2]. Overuse, or early stage tendinopathy due to excessive loading, can cause microtraumas with eventual chronic degenerative changes. It still remains controversial as to whether or not inflammation plays a role in early stage tendinopathy [44]. Some studies have suggested that is unwarranted to have treatment processes for tendinitis, or inflammation of the tendon in early stage injury, as most overuse injuries present with no obvious inflammation [2,45]. However, other studies analyzing human biopsies have presented evidence that there may be a brief inflammatory response in early tendon injury, suggesting that inflammation and degeneration may not be mutually exclusive [44].

Still, the etiology of overuse injury is not well understood, but is considered multifactorial [1,2,40,41,46]. Accepted intrinsic factors include 1) excessive mechanical

loading that leads to ECM damage and morphological changes of resident tenocytes, 2) imbalances in the proteases that degrade ECM components and their endogenous inhibitors, and 3) other individual factors such as age, gender, anatomy, and genetic disorders [2,43,44,47].

Tendinopathy is a common disorder that can occur in any tendon, including plantar fasciitis, Achilles tendinopathy, patellar tendinopathy, tennis elbow, golf elbow, and rotator cuff tendinopathy [2] Tendinopathy accounts for about 50% of all sports injuries, occurring in 30% of all running related injuries, and in 40% of tennis players [48–50]. Rotator cuff injuries are the most prevalent cause of shoulder injury, and pain in the shoulder is the 3rd most common orthopaedic problem presented to clinicians [51,52]. In addition to athletes, rotator cuff injuries commonly affect laborers and aging adults [1,53]. It is the most common musculoskeletal problem for people over 65 yrs [40,54]. Rotator cuff tendon tears present in about 13% of people in their 50s, 25% of people in their 60s, and 50% of people in their 80s [45,46]. Without repair tendon tears can lead to damage to surrounding tissues, including muscle atrophy and fatty infiltration, cartilage degeneration, and altered joint biomechanics [45,52]. Between 30,000-75,000 rotator cuff tendon repair surgeries are performed each year [45]. Depending on patient age and the level of degeneration that occurred before surgical intervention, single-tendon and two-tendon re-tear rates can be between 29 and 94% despite patients having excellent surgical outcomes [50,52].

The rotator cuff is composed of 4 muscle-tendon complexes: the supraspinatus, infraspinatus, subscapularis, and teres minor. These tendons insert onto the humeral head and are responsible for transmitting muscle-generated forces to the humeral bone that induce movement. The tendons also function to provide stability to the glenohumeral joint [2,40,53]. Type I collagen makes up 95% of the total collagen content in tendon,

with small amounts of other types of collagen, proteoglycans, and resident tenocytes—elongated and spindle shaped cells sparsely distributed throughout the tissue that align with the collagen [1,2,41]. Tendon is very linearly organized in a hierarchical structure that begins with the smallest unit, type I collagen. Collagen molecules organize into crimped fibrils, that then self-assembles as fibers, followed by primary, secondary, and tertiary fascicles, and then the full tendon unit [2,40,42] (Fig 2.1).

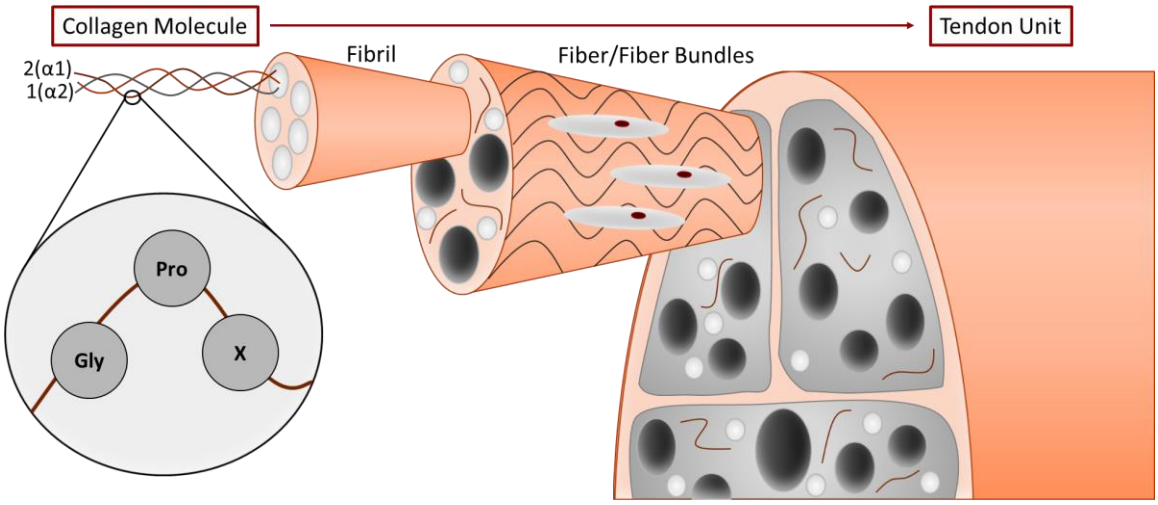

Figure 2.1. Schematic of tendon hierarchical structure.

The proteoglycans bound to the collagen fibrils have a protein core and polysaccharide glycosaminoglycan (GAG) side chains. These hydrophilic molecules contribute to the viscoelastic mechanical properties of tendon, lubricating the tissue, allowing fluid movement [40–42]. The hierarchical structure and crimping nature of the

collagen-dense tendon is what allows for the support of high forces, as fascicles slide past one another in response to mechanical loading [40,43,45,55].

Cathepsins and MMPs are major players in facilitating ECM remodeling and degradation of matrix proteins that lead to degeneration. Thus, in this study, elucidating the role of cathepsins and MMPs as part of the underlying mechanisms contributing to early stage tendon injury is important for developing interventions to prevent rupture.

2.3 Complexity of the Proteolytic Network: Cathepsins and Matrix Metalloproteinases

Proteolysis, the irreversible hydrolysis of covalent peptide bonds in a protein sequence, is involved in many cellular processes that dictate normal function and remodeling in all tissue systems [6]. Due to their destructive potential, proteases are highly regulated by endogenous inhibitors, pH, and activation of zymogens to mature forms [6,11,13,16,56,57]. However, imbalances in protease activity and inhibition lead to abnormalities, such as altered cell function, apoptosis, and tissue degradation [6,56,57]. These proteases and inhibitors are all present intracellularly and can be secreted into the extracellular space. As a result, these proteins are able to interact by directly or indirectly activating, inhibiting, or enhancing other proteolytic functions, thus contributing to a very complex and dynamic proteolytic network [58].

In addition to cysteine cathepsins, matrix metalloproteinases (MMPs) are a proteolytic family whose upregulation, and the downregulation of their endogenous inhibitors, have been implicated in numerous disease states, including osteoarthritis and osteoporosis, and rotator cuff rupture [3,5,13,40,59–63]. MMPs are a family of 25 zinc-dependent endopeptidases, most highly expressed in mammals. They are capable of

degrading a variety of ECM components and are generally active at a neutral pH [3,4,21,64]. Both cathepsins and MMPs are translated and secreted as inactive zymogens that must be activated in order to cleave their substrates. Cathepsins (K, L, S, and V) and MMPs (1, 2, 8, 9, 13, 14) are the proteases most implicated in the degradation of type I collagen (the main component in tendon), but cathepsin K most potently degrades collagen. Cathepsins and MMPs, in addition to type I collagen, are known to degrade other ECM proteins as well, such as other collagens, proteoglycans, and cell adhesion molecules [4,6,21,42,65]. The endogenous inhibitors, cystatins (cathepsins) and tissue inhibitors of metalloproteinases (TIMPS), are also highly regulated and can act intra- or extracellularly to maintain homeostasis and prevent progression to pathological states [3,4,6,13,57,66,67].

The activation and inhibition, or crosstalk, between these proteins regulate activation, activity, and ECM cleavage even across families and play key roles in interfamilial proteolytic function [58]. Cathepsin K has been shown to active and enhance MMP-9 activity in human osteoclasts [68]. Our lab has demonstrated protease-on-protease hydrolysis, or cannibalism, between cathepsins even in the presence of a substrate. Less immunodetectable recombinant cathepsin K was observed over a 2hr period when it was incubated with elastin, indicating autodigestion, or cleavage of itself. Cathepsin S preferentially degraded cathepsin K after 2hrs even when incubated with collagen or elastin, and thus, diminished the substrate cleavage potential [69]. Furthermore, we have also demonstrated that cathepsin family members can have drastically different reactions to broad spectrum inhibition. Treatment of E64, a broad spectrum small molecule cathepsin inhibitor, on MDA-MB-231 breast cancer cells

increased the amount of active cathepsin S while simultaneously decreasing the amount of active cathepsin L after 24 hrs [70]. These studies all illustrate the dynamic complexity of the interactions between and within cathepsin and MMP proteolytic families, in addition to their inhibitors. Because cells are able to secrete multiple proteases simultaneously, the cooperative or antagonistic interactions between them is important for understanding how proteolytic upregulation modulates ECM degradation.

2.4 Analysis of Proteolytic Actions by Cathepsin K and Matrix Metalloproteinases on ECM

Cathepsin K has a unique ability to cleave insoluble collagen as compared to MMP-1, -9, or -13. Demineralized bone particles were incubated with cathepsin K, MMP-1, -9, and -13, over 96 hrs and degradation was compared to that resulting from bacterial collagenase [21]. This study used hydroxyproline spectrometry analysis to quantify the amount of collagen solubilized by these enzymes normalized to collagenase. Type I collagen digestion and subsequent sequence analysis also revealed cleavage differences in MMPs, less collagenolytically potent cathepsins, and cathepsin K [21]. MMPs are able to cleave intrahelically through the collagen molecule to form $^{3/4}$N-terminal and $^{1/4}$C-terminal fragments that can be further degraded by other enzymes [3], and cathepsin L is only able to cleave at the telopeptide ends.

Cathepsin K, however, is able to cleave not only at the telopeptide ends, but also at multiple points within the matrix. Cleave sites were identified 9 and 21 amino acids from the N-terminal end of the helical domain in both α1 and α2 chains, 96 and 810 amino acids from the N-terminal end in the α1 chain, and 99 and 814 amino acids from the N-terminal end in the α2 chain. This was further supported by the collagen

fragmentation analysis using enzyme-linked immunosorbent assays (ELISA) specific for C-terminal (CT_x) and N-terminal (NT_x) telopeptide fragments, present in the solubilized collagen supernatant. The study found that cathepsin K was able to solubilize significantly more collagen than any of the MMPs at 24, 48, 72, and 96 hr time points. Cathepsin K was also able to generate substantial CT_x and NT_x fragments while the MMPs were not [21]. In a subsequent study published in 2003, it was found that while cathepsin K released large amounts of CT_x fragments from type I collagen, the MMPs released cross-linked carboxyterminal telopeptide of type I collagen (ICTP) fragments and no detectable amount of CT_x fragments [71,72] (Fig 2.2). These studies demonstrate the clearly unique collagen cleavage patterns between catK and collagenolytic MMPs, and suggest that the actions of catK may be more important when assessing collagen degradation in tissue contexts.

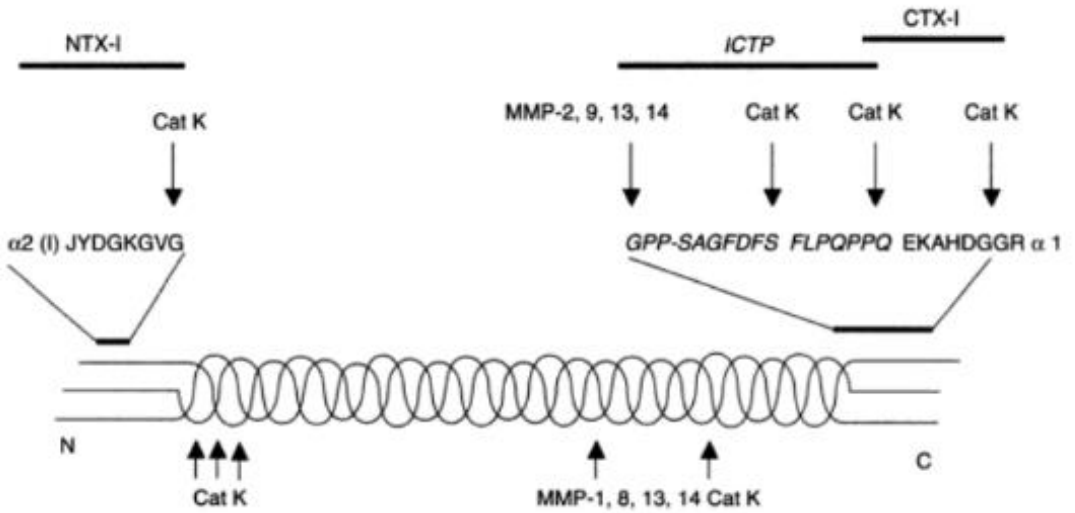

Figure 2.2. Schematic of type I collagen peptides generated by cathepsin K (CTX & NTX) and MMPs (ICTP). Adapted from Moskowitz et al. 2007.

Another study published work researching the action of recombinant cathepsins on proteoglycan and insoluble collagen degradation. Their work suggested, not only that cathepsins cleave more effectively in the presence of GAGs [25], but also that collagen-bound GAGs serve as docking points in the gaps between molecules for cathepsin K to bind and form a dimer. The study conjectured that the formation of this complex forced the triple helical collagen molecule open and allowed access to the binding points on the α-chains and allowed for collagen cleavage by other proteases [42,73]. This would suggest that maintaining adequate GAG content within the collagen fibers is necessary to facilitate cleavage by cathepsin K.

The ubiquitous expression of many cathepsins and their potency in degrading ECM has made the development of cathepsin deficient models advantageous for studying many pathologies [74–76]. The development of cathepsin K deficient mouse models allows investigation of its effects in different tissue and organ systems and identify potential therapeutic targets for the overexpression of cathepsin K in disease states [6,8]. Cathepsin K deficiency ultimately shows reduced capacity for matrix remodeling. These mice exhibit osteopetrosis—dense but fragile bone structure—with altered morphology and diminished resorptive activity of osteoclasts. The mice also presented with impaired vascular repair, severe cognitive abnormalities, lung fibrosis, open fontanels, and other bone deformities [74,77]. Interestingly, however, these cathepsin K knockout mice also were protected from atherosclerotic and arthritic development. It has also been shown that in cathepsin K deficient mice osteoclasts numbers increase and other proteases rescue some of the bone resorption lost by the lack of cathepsin K, though to a lesser degree of remodeling [71,77]. The cathepsin K deficient animal model provides the

unique context to study how the proteolytic network adapts to the loss of catK in the network of interactions—specifically, it allows the study of how the accumulation of ECM components impacts biochemical and biomechanical cues between matrix and cells. Cells may upregulate other proteases or downregulate inhibitors in order to recover matrix remodeling capabilities in the absence of the most potent collagenase. Furthermore, because cathepsin expression differs between tissues, localized cellular changes may reveal the necessity of catK in remodeling of specific tissues.

2.5 Proteases in Early Stage Rotator Cuff Tendinopathy

Many animal models, specifically in rats, rabbits, and sheep, have been developed to gain fundamental knowledge on the mechanisms of tendon pathogenesis to varying degrees of success [46]. In 2000, the Soslowsky lab developed a rat model of rotator cuff tendinopathy using a downhill treadmill running regimen to induce tendinopathic damage [47]. With rats having a similar coracoacromial arch anatomy to humans, running downhill recapitulated human injury as closely as possible, making this model advantageous for fundamental research [78]. Using this model, the Soslowsky group was able to induce histopathological and mechanical changes similar to the observed degeneration from human tissues [47].

MMPs have been implicated in tendon ECM remodeling [2,4]. Specifically in early stage tendinopathy, MMP-2 and MMP-14 have demonstrated increased mRNA and active protease levels in a rat overuse model similar to the one developed by the Soslowsky lab [79]. Cysteine cathepsins have been implicated in tissue destructive diseases such as cancer, osteoarthritis, and osteoporosis [13,59,60]. Our lab has published a temporal upregulation of catK and catL in a rat model of rotator cuff overuse adapted

from the Soslowsky model. After 4 weeks of injury active catK and catL were upregulated, but by 8 weeks, only active catL remained upregulated [5]. The upregulation of multiple proteases, in our study as well as others, has motivated further investigation of the roles of both cathepsin and MMPs in cleaving tendon ECM degradation in order to suggest potential intervention strategies.

CHAPTER 3. Supraspinatus Tendon Overuse Results in Degenerative Changes to Tendon Insertion Region and Adjacent Humeral Cartilage in a Rat Model

This chapter was adapted from Parks et al. (2016). Supraspinatus tendon overuse results in degenerative changes to tendon insertion region and adjacent humeral cartilage in a rat model. Journal of Orthopaedic Research, 35(9), 1910-1918. doi: 10.1002/jor.23496 [80]

Elements of this chapter were also adapted from Seto et al. (2015). Cathepsins in rotator cuff tendinopathy: identification in human chronic tears and temporal induction in a rat model. Annals of Biomedical Engineering, 43(9), 2036-2046. doi: 10.1007/s10439-014-1245-8 [5]

3.1 Introduction

Rotator cuff tendon overuse, or tendinopathy, is a common degenerative disorder that can result in pain and limited range of motion [1,53]. This injury particularly affects athletes and laborers using a repetitive overhead motion, as well as older adults [1,53]. Tendinopathy is characterized clinically by changes in tenocyte phenotype, disorganization and misalignment of collagen fibers, and decreased mechanical properties [1,47]. If left untreated, overuse injuries can predispose tissue to full or partial-thickness tears, requiring surgical intervention [2]. Thus, elucidating underlying mechanisms of early tendon injury is important to develop new interventions to prevent tendon rupture.

Though the etiology of overuse is multifactorial, accepted intrinsic factors of tendinopathy include both excessive mechanical loading that leads to microtears and decreased loading and morphological changes of resident tenocytes, as well as imbalances in proteases that degrade extracellular matrix (ECM) components [2,43,78].

Although proteolytic imbalances have been implicated in rotator cuff tendon ECM degradation, how proteases cause deterioration in early tendon damage is not well understood [45,46]. In previous work, our laboratory demonstrated the clinical significance of cathepsins in end stage supraspinatus tendon injury. Zymography on debrided human supraspinatus tendon tissues collected during reconstructive surgery showed active cathepsins K, V, S, and L in 5 of the 6 patient samples (Fig 3.1) [5].

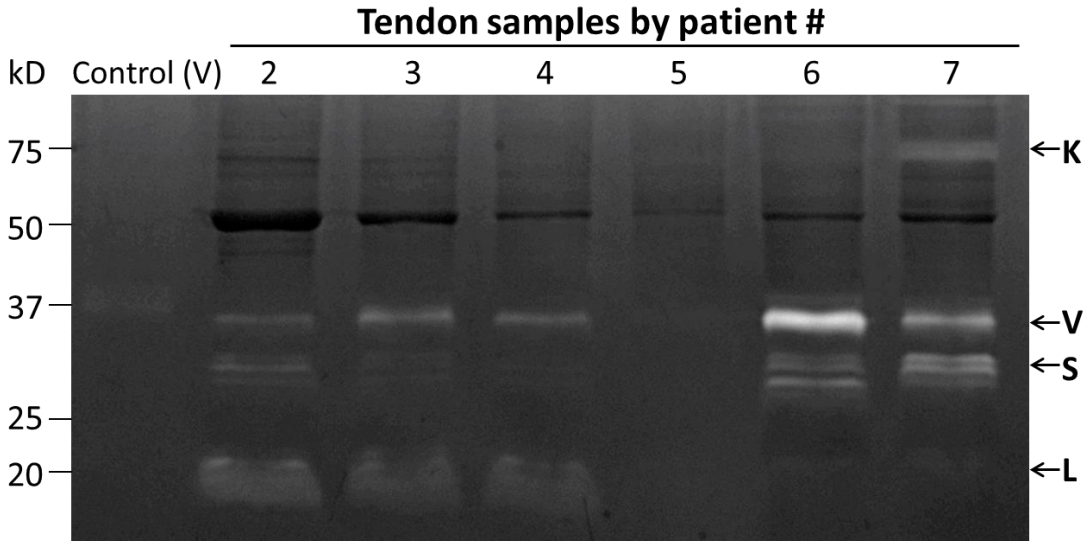

Figure 3.1. Cathepsin activity in human chronic rotator cuff tendon tears. Zymogram depicts activity of cathepsins K, V, S, and L. Adapted from Seto et al. 2015.

Additionally, we showed that after 4 and 8 weeks of rat supraspinatus tendon overuse, cysteine proteases cathepsins K and L were upregulated at the tendon insertion region (17% of length from the osseotendinous junction) when compared to their age-

matched controls [5]. This finding of cathepsin upregulation only near the insertion region motivated the current study to focus on protease activity in tissues near the humeral head, notably the tendon insertion and the humeral articular cartilage. We investigated tissue degeneration in these areas using a well-established rat model of supraspinatus tendon overuse that displays similar bony anatomy to the human rotator cuff [5,47,50,78,81,82]. Because our previous experiments focused on tendon damage at 4 and 8 weeks of overuse, showing cathepsins K and L upregulated at 4 weeks and only cathepsin L upregulated at 8 weeks, we have expanded this time course to investigate damage at earlier (2 weeks) and later (10 weeks) time points of tendon overuse in order to identify the time of greatest tissue damage and peak proteolytic activity.

Several studies have implicated cathepsin K, the most powerful mammalian collagenase [21] in several tissue destructive diseases, including cancer, osteoporosis, and osteoarthritis (OA) [59,60,83]. Human cathepsin V is the most potent mammalian elastase identified, but also possesses collagenolytic activity and is upregulated in cancer and autoimmune disorders [8,24,25]. Its ortholog, murine cathepsin L [15], is upregulated in diabetic mice at mRNA and protein expression levels and contributes to cardiovascular disease [24,26]. As demonstrated in our previous work [5], cysteine cathepsins K and L are active and may result in collagen degradation in tendon overuse injury. In particular, it is known that cathepsin K can cleave collagen at both telopeptide ends, and can also cleave intrahelically, making collagen more susceptible to further degradation by other proteases [21].

Moreover, cathepsins are also capable of cleaving and activating matrix metalloproteinases (MMPs) [68]. Thus, examining the proteolytic contributions of these

two protease families in tandem could provide valuable mechanistic information about ECM degradation in tendon. MMPs are a family of zinc-dependent endopeptidases that can degrade and remodel tendon ECM [4,47]. A prior study demonstrated upregulation of MMP-1 activity and down regulation of MMP-2 and -3 activity in ruptured human supraspinatus tendon [3] while another found upregulation of MMP-2 mRNA and protein activity and MMP-14 mRNA in a rat model of overuse [79], similar to the model used in the current research. These findings confirm that MMPs are present during injury and may be contributing to tendon degeneration in addition to cathepsins.

Rotator cuff tendons provide stability to the glenohumeral joint, but tears can alter joint loading and can induce cartilage degeneration [2,45,84]. Several studies in rats have found evidence of osteoarthritic-like changes in cartilage after tendon transection (modeling full tendon tears) [81,85,86]. However, it remains unclear how tendon overuse injury affects nearby articular cartilage, both in terms of structural damage and proteolytic activity. Cathepsin K has been found to be upregulated in osteoarthritic cartilage and synovial tissues [59]. Additionally, MMP-2, -9, and -13 have also been implicated in human and rat cartilage degeneration [87,88]. Thus, we analyzed articular cartilage on the humeral head using safranin-O staining and Equilibrium Partitioning of Ionic Contrast agent via micro-computed tomography (EPIC-μCT) [89] after 10 weeks of treadmill running to determine if supraspinatus tendon overuse would result in cartilage degeneration, as well as examined protease activity in this tissue.

Overall, the objective of this study was to characterize the level of tissue damage and proteolytic activity in supraspinatus tendon insertion tissues after 2 and 10 weeks of rat supraspinatus overuse, and also to determine if this model yielded tissue damage in

humeral articular cartilage and subchondral bone, or proteolytic upregulation in humeral articular cartilage. Based on previous results, as well as their ability to activate MMPs, it was hypothesized that the greatest cathepsin upregulation in the supraspinatus tendon insertion tissues would occur at the earliest point of overuse. It was also hypothesized that both supraspinatus tendon insertion and cartilage tissues would show evidence of damage and proteolytic upregulation at 10 weeks of overuse.

3.2 Methods

3.2.1 Rat Overuse Model

All animal procedures in this study were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. 68 male Dahl Salt-Resistant rats (~350 g initial weight, 14-15 weeks initial age) were obtained (Harlan Labs, Indianapolis, IN), chosen because they are an inbred strain derived from the outbred Sprague-Dawley strain used to develop the injury model and have previously been used in work published by our group [5]. Animals were separated into 2 or 10 week control and experimental groups. Experimental rats were subjected to a downhill treadmill rotator cuff overuse regimen as described previously [47]. 34 animals were trained for 2 weeks, running at a 10° decline for 1hr/day for 5days/week increasing speeds up to a final 17 m/min. Rats were then subjected to overuse for 2 or 10 weeks (n=13/group/time point). Age-matched control rats were allowed normal cage activity (n=13/group/time point). At each time point, control and overuse animals were sacrificed and supraspinatus tendons were harvested from animals designated for tendon histological and biochemical analysis. For biochemical analysis, the insertion region of the tendon (first 17% of the length from the osseotendinous junction) was isolated prior to analysis. Animals designated for

tendon mechanical testing and cartilage biochemical analysis were sacrificed at 10 weeks and prepared for further processing.

3.2.2 Tendon Histology and Scoring

For histological evaluation, tendons were embedded in optimum cutting temperature (OCT) and frozen in a liquid nitrogen-chilled ethanol slurry. Tendon tissues were sectioned into 10 μm longitudinal sections using a cryostat (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA). Slides were stained with hematoxylin and eosin (H&E) (VWR, Radnor, PA) and the insertion area of each tendon was imaged with a Nikon TE2000. H&E stained sections were histologically graded using a semi-quantitative scale, similar to the Bonar and Movin scales [90]. Within each time point, the control and experimental groups were compared and scored in each of the following categories: regional variations in cellularity, cell shape, collagen fiber organization, and vascularization. A 4-point scoring system was used, where 0 indicated a normal tendon appearance and 3 a markedly abnormal appearance. Five graders, blinded from experimental group and time point, (TR, LT, JL, MO, JK) scored 4 images from each tendon (n=8-9/group/time point). Scores were ranked and analyzed as individual events for statistical analysis (see "statistical analysis" section).

3.2.3 Tendon Mechanical Testing

Following a well-established protocol for mechanical testing [47,91], supraspinatus tendons (n=10/group/time point) were cleaned of extraneous tissue and the humeral bones were mounted into custom-designed acrylic rings using Ortho-Jet BCA acrylic resin (Lang Dental, Wheeling, IL) and left in phosphate-buffered saline (PBS) overnight to polymerize. Subsequently, tendon width and thickness were measured at the

approximate middle point along the length of the tissue using calipers. Prior to testing, the free end of each tendons was clamped with fine grit sandpaper and marked every 2mm, beginning at the insertion, using India Ink. Tendons were submerged in a 37°C PBS bath and preconditioned for 10 cycles. Samples were loaded to failure using a 100N load cell at a constant 24μm/s rate on a MTS Mini Bionix II system. Testing was captured using a Manta G504B ASG digital Camera (Graftek Imaging, Austin, TX) and recorded using LabView. Measurements for determining insertion moduli were taken by analyzing displacement between the two lines closest to the insertion region in ImageJ (NIH).

3.2.4 Second Harmonic Generation Imaging

Second harmonic generation (SHG) imaging was performed to asses for tendon fibrillar structure as previously described [55,92]. For SHG imaging, tendons subjected to overuse for 10 weeks and their age-matched controls (n=10/group) were embedded in optimum cutting temperature (OCT) and frozen in a liquid nitrogen-chilled ethanol slurry. Tendon tissues were sectioned into 30 μm longitudinal sections using a cryostat (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA). The insertion area of each tendon was imaged with a Zeiss 710 non-linear optics (NLO) confocal microscope using an 800 nm excitation wavelength and emission wavelengths between 380 and 420 nm. Second harmonic was picked up by photomultiplier tubes to generate images. Images were visually analyzed for fiber organization, density, and consistency in crimping across the tissue, used to indicate collagen damage in each tendon sample.

3.2.5 Cartilage micro-computed tomography (μCT) and Equilibrium Partitioning of Ionic Contrast Agent via μCT (EPIC-μCT)

For microcomputed tomography, samples (n=8/group/time point) were analyzed by μCT and EPIC-μCT based on established techniques [89]. Following sacrifice, shoulder joints were scanned using viva CT (Scanco Medical, Brüttisellen, Switzerland) at 55kVp, 142μA, and a 200ms integration. Four distinct points along the humeral head were chosen for analysis based on 3 subdivided cartilage regions analyzed previously [86]. Joint gap thickness was measured at these four points for both control and overuse animals. Subsequently, humeral heads from the same shoulders were detached from remaining humeral bone and tendons at the insertion region and submerged in a 10% solution of Hexabrix 320 ionic contrast agent (Covidien, Dublin, Ireland) at 37°C for 30 minutes. Humeral heads, oriented based on the location of the removed tissue, were scanned in the sagittal plane with the μCT50 (Scanco Medical, Brüttisellen, Switzerland) at 45kVp, 200μA, and a 600ms integration time. The Hexabrix attenuation in cartilage at equilibration is inversely correlated to glycosaminoglycan (GAG) content, which is lost in cartilage degeneration [93]. Following segmentation and reconstruction of the 3D images, cartilage thickness, cartilage attenuation, and subchondral bone thickness and attenuation were measured at four points along the humeral head for both control and overuse animals using μCT50 software.

3.2.6 Cartilage Histology

Scanned humeral heads (n=8/group/time point) were decalcified by submerging them in Cal-Ex II (Thermo Fisher Scientific) for 1 week, exchanging solution every other day. After complete decalcification was achieved (as verified by a single X-ray scan

using the viva CT), humeral heads were rinsed with PBS and embedded in OCT and frozen in a slurry of liquid nitrogen-chilled ethanol. The tissue was sectioned to 10μm using a cryostat (CryoStat NX70). Slides were stained with 0.5% safranin-O and 0.2% fast green stains (Sigma Aldrich, St. Louis, MO) and imaged using a Nikon TE2000 to observe sulfated GAG content.

3.2.7 Multiplex Gelatin Zymography

In preparation for zymography, the insertion region of each supraspinatus tendon (n=6/group/time point) was isolated, and humeral articular cartilage (n=7/group/time point) was carved from the humeral head. Tissues were homogenized in a zymography lysis buffer with 0.1mM leupeptin, and the supernatant was collected. Multiplex zymography for cathepsins and MMPs was performed as previously described [83,94]. Protein concentration in each lysate sample was determined by a micro BCA kit (Thermo Fisher Scientific). 11μg of protein from each sample was loaded into 12.5% (cathepsin) and 10% (MMP) SDS-polyacrylamide gels embedded with a 0.2% gelatin substrate. Using electrophoresis, enzymes separated by molecular weight. Then the gels were washed in renaturing buffer to remove the SDS and allow the enzymes to return to their active confirmation. Gels were then incubated in pH4 cathepsin assay buffer or pH7.4 MMP assay buffer for 18hrs at 37°C to allow the proteases to degrade the gelatin. Subsequently, gels were stained with Coomassie Blue and destained to reveal white bands indicative of active proteases. Gels were then imaged using the ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, United Kingdom).

Densitometry analysis was performed on the acquired images using ImageJ. Bands in zymography gels comparing control and overuse supraspinatus insertion

samples at each time point were normalized to 1ng of recombinant mouse cathepsin L (R&D Systems, Minneapolis, MN) and 0.01ng recombinant human MMP-2 (ENZO, Farmingdale, NY). Zymography bands from cartilage samples were normalized to the control averages for each gel.

3.2.8 Statistical Analysis

The sample sizes were chosen based on power analysis performed on data from our previously published work [5]. The power analysis revealed a minimum n=5 for tendon and cartilage zymography with α=0.05 and β=0.15. The power analysis showed a required n=8 with α=0.05 and β=0.2 for tendon histology and n=5 with α=0.05 and β=0.2 for tendon mechanical testing. The power analysis revealed a minimum n=8 for cartilage μCT imaging with α=0.05 and β=0.05. Histological scoring of tendon insertions was evaluated by the non-parametric Mann-Whitney test using Minitab. Data from the Mann-Whitney analysis was presented as rankings between 0 and 2 based on the frequency of each score value. Statistical significance for μCT data was determined by a Student's t-test in Minitab. Statistical significance for tendon zymography data was determined using a two-way ANOVA between normalized control and overuse data in GraphPad. Statistical significance for all other data was determined by a Student's t-test between normalized control and overuse data with a $p<0.05$ using Microsoft Excel.

3.3 Results

3.3.1 Supraspinatus tendon insertion histology

Histological staining of 2 week control supraspinatus tendon insertions showed minimal damage indicated by aligned collagen fiber structure with elongated, spindle-

shaped cells (Fig 3.2A). Similarly, the 2 week overuse tissues demonstrated very little evidence of damage with minimal fiber disruption and elongated cells (Fig 3.2B). At 10 weeks, control tissues maintained similar levels of tissue structure with aligned, tightly packed fibers and a slight mix of elongated and rounded cells (Fig 3.2C). Only in the 10 week overuse tissues, however, was there evidence of cellular rounding, collagen fiber misalignment, and fiber thinning, consistent with tendinopathic histological changes (Fig 3.2D).

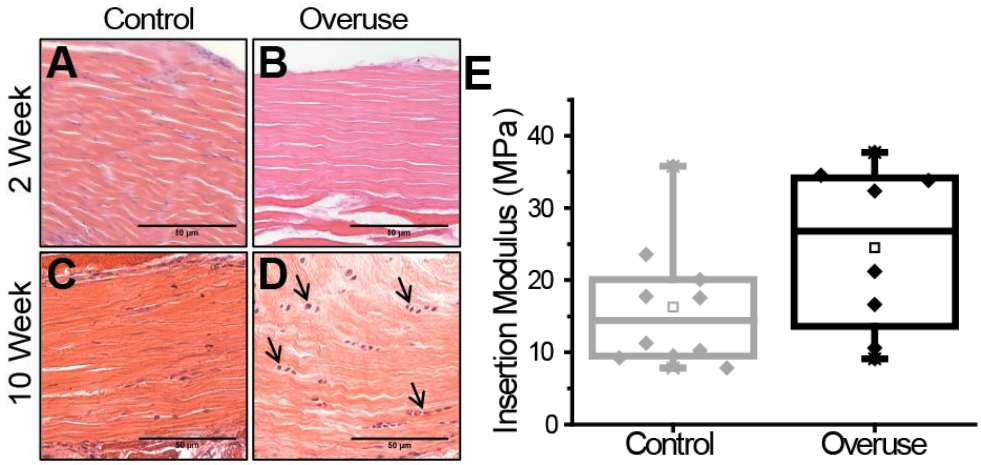

Figure 3.2. Histological and mechanical assessment of supraspinatus damage at 2 and 10 weeks of overuse. Collagen fiber thinning, disorganization, and cell rounding were seen in the supraspinatus insertion by 10 weeks of overuse. The insertion region for 2 week (A) and 10 week control (C) animals are contrasted with the insertion region of 2 week (B) and 10 week overuse (D) tendons (n=8-9). Arrows indicated rounded tendon cells. Tensile testing of the supraspinatus insertion regions of both control and overuse animals showed no significant difference (E) (SD: control: ±8.7MPa; overuse: ±11.5MPa) (n=10).

Analysis of histological scoring reported decreased variation in cellularity at 2 weeks in the overuse compared to the controls, while there were no significant differences in scores for cell shape, collagen fiber organization, or vascularity at that time point. Additionally, only in 10 week overuse animals did tendons receive significantly higher scores for cell shape, indicating cell rounding, and in collagen fiber organization, indicating a disruption in the collagen fiber arrangement. Neither regional variation of cellularity nor vascularization received scores that were statistically different at 10 weeks in either experimental group (Table 3.1).

Table 3.1. Histological scoring analysis of control and overused supraspinatus tendon insertion regions.

	2 week		10 week	
Category	**Control**	**Overuse**	**Control**	**Overuse**
Regional Variation of Cellularity	1*	0	0	0
Cell Shape	0	0	0	1*
Collagen Fiber Organization	1	1	0	1*
Vascularization	0	0	0	0

* Denotes significant difference (n=8-9).

3.3.2 Supraspinatus tendon insertion mechanical testing

No significant difference was found in cross-sectional area between tendons from overuse animals (0.66±0.14 mm^2) vs. control (0.77±0.16 mm^2). Similarly, analysis of mechanical testing results showed no significant differences in the tensile moduli between overused (24.5±11.5 MPa) and control (16.3±8.7 MPa) tissues (Fig 3.2E).

3.3.3 Second harmonic generation imaging of supraspinatus tendon collagen structure

SHG imaging on supraspinatus insertion regions of rats subjected to overuse for 10 weeks revealed obvious disturbances in the crimping pattern with indications of fiber separation and collagen disorganization in the insertion region (Fig 3.3B). By contrast, the age-matched control images show aligned, intact fibers with crimping across the tissue.

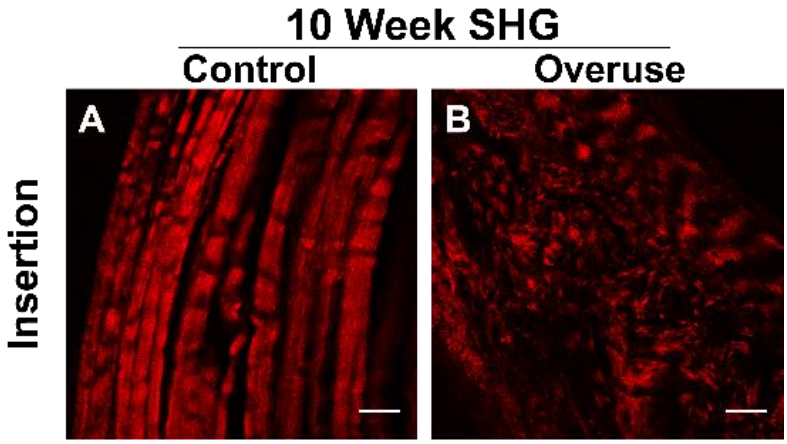

Figure 3.3. Second harmonic generation imaging of supraspinatus tendon insertion region after 10 weeks of overuse. SHG images displaying changes in fibrillar structure after 10 weeks of overuse in the insertion region of the supraspinatus tendon. The crimping pattern across the tissue in the overuse insertion region appears more disturbed (B) than the control (A). n=10, scale bar = 50 μm.

3.3.4 EPIC-µCT & histological analysis of humeral articular cartilage and subchondral bone

Joint gap distances determined from reconstructed models of the rat glenohumeral joint were significantly reduced in running animals (527.3±16.3µm; 264.3±8.9µm; 291.9±40.7µm; 263.8±29.4µm) in comparison to their age-matched controls (616.8±35.9µm; 366.4±32.7µm; 395.1±68.8µm; 336±41.9µm) along the four points on the humeral head (Fig 3.4).

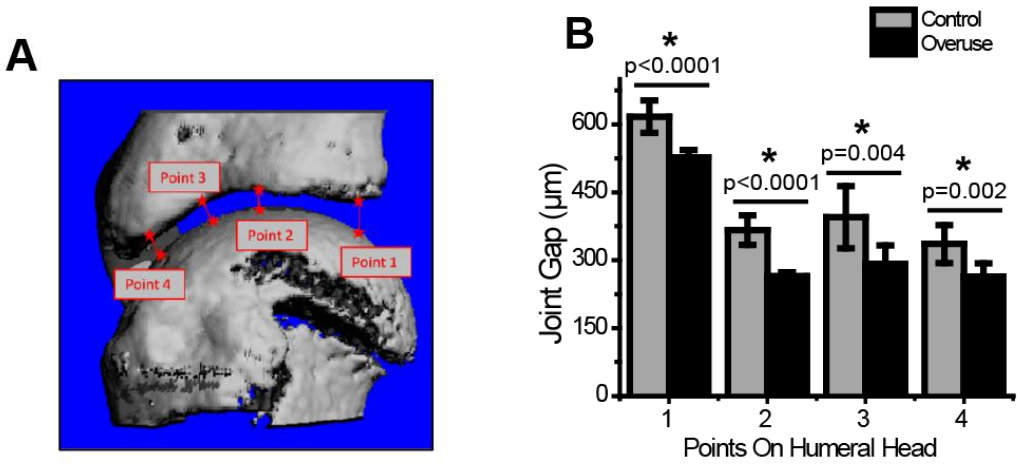

Figure 3.4. µCT measurements of glenohumeral joint gap. Joint gap thickness as measured by µCT along 4 different points of the glenohumeral joint (A) showed decreased joint space in overuse animals as compared to age-matched controls (B) (SD: control: ± 35.9, 32.7, 68.8, 41.9µm; overuse: ±16.3, 8.9, 40.7, 29.4µm). * denotes significant difference, p-values as stated (n=8).

Measurements from EPIC-μCT scans revealed that cartilage thickness was decreased with experimental animals (119.6±6.34μm; 93.2±10.8μm; 74.1±10.4μm; 56.9±6.43μm) compared to their age-matched controls (195.4±13.4μm; 141.6±7.68μm; 111.1±11.9μm; 90.1±9.63μm) at each of the four points analyzed (Fig 3.54A,B,C). EPIC-μCT also revealed decreased GAG content, as indicated by the significant increase in contrast dye attenuation (overuse: 2.1±0.18cm^{-1}; control: 1.65±0.14cm^{-1}) (Fig 3.5B,C,F), which was confirmed with safranin-O staining of the decalcified humeral heads (Fig 3.5D,E). Subchondral bone thickness was higher in overuse animals (216.2±10.9μm) compared to control animals (192±17.8μm) (Fig 3.5G). However, there was no difference in mineral density of the subchondral bone between the two groups (overuse: 4.79±0.06cm^{-1}; control: 4.78±0.09cm^{-1}) as demonstrated by quantification of subchondral bone attenuation (Fig 3.5H).

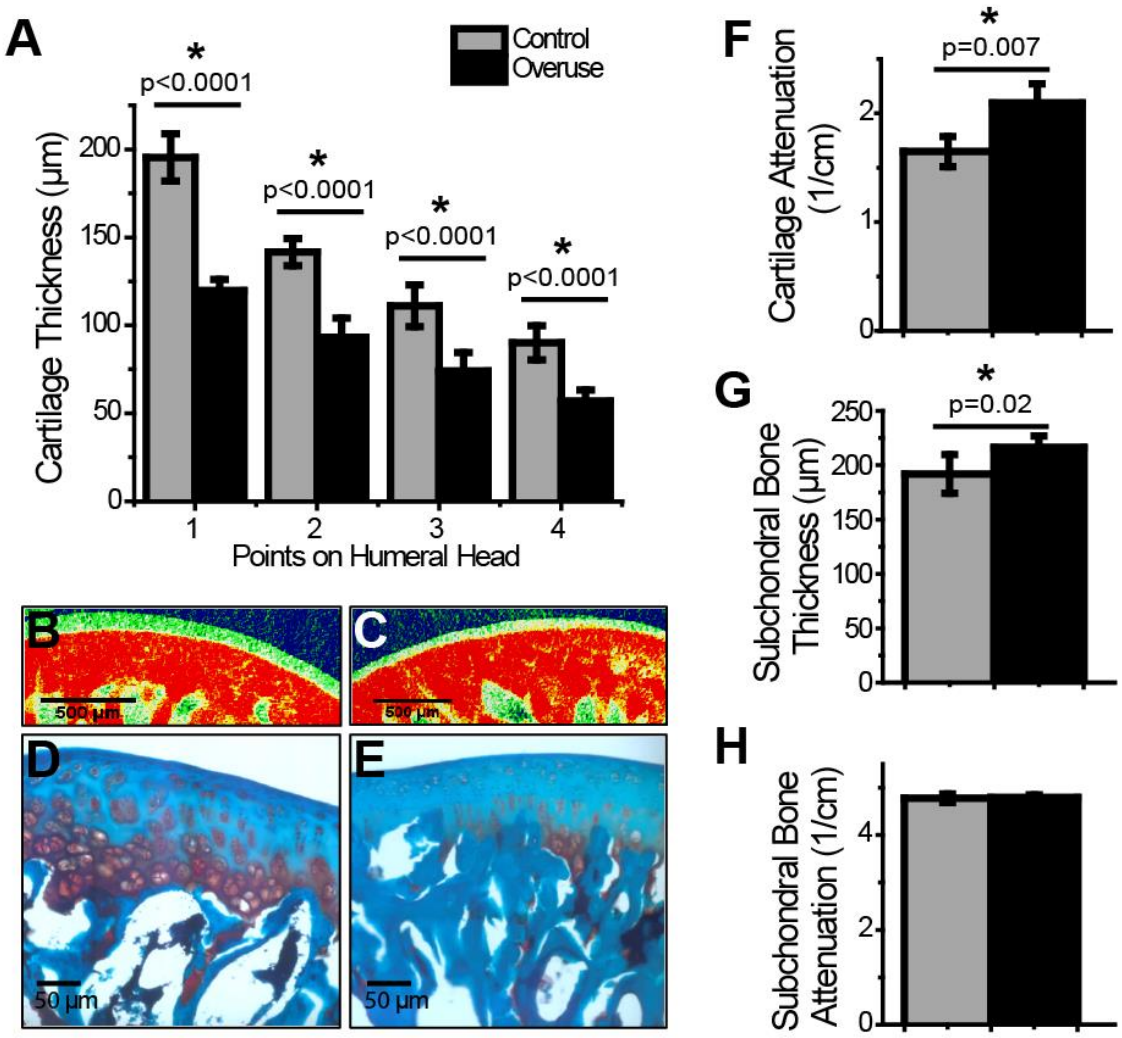

Figure 3.5. EPIC-µCT measurements of humeral articular cartilage and subchondral bone. Humeral heads demonstrated a reduced cartilage thickness in overuse animals in comparison to their age-matched controls (A) (SD: control: ±13.4, 7.7, 11.9, 9.6µm; overuse: ±6.3, 10.8, 10.4, 6.4µm). Humeral cartilage also displayed decreased GAG content by EPIC-µCT in overuse animals in comparison to control (B, C, F) (SD: control: ±0.14cm-1; overuse: ±0.18cm-1) which was confirmed with Safranin-O staining of the decalcified humeral head (D, E). While subchondral bone thickness was higher in overuse animals as compared to controls (G) (SD: control: ±17.8µm; overuse: ±10.9µm), there was no difference in mineral attenuation in the subchondral bone (H) (SD: control: ±0.09cm-1; overuse: ±0.06cm-1). * denotes significant difference, p-values as stated (n=8).

3.3.5 Cathepsin and MMP activity in supraspinatus tendon insertion

After 2 and 10 weeks of overuse, active cathepsin L was detected in the pH 4 cathepsin zymograms between 25 and 35kD, while active pro- and mature MMP-2 were detected in pH 7.4 MMP zymograms around 68 and 72kD, respectively, in rat supraspinatus insertions of both overuse and control animals. Normalized densitometric analysis revealed no significant differences in the amounts of either active cathepsin L or MMP-2 between after 2 or 10 weeks of overuse (Fig 3.6A,B).

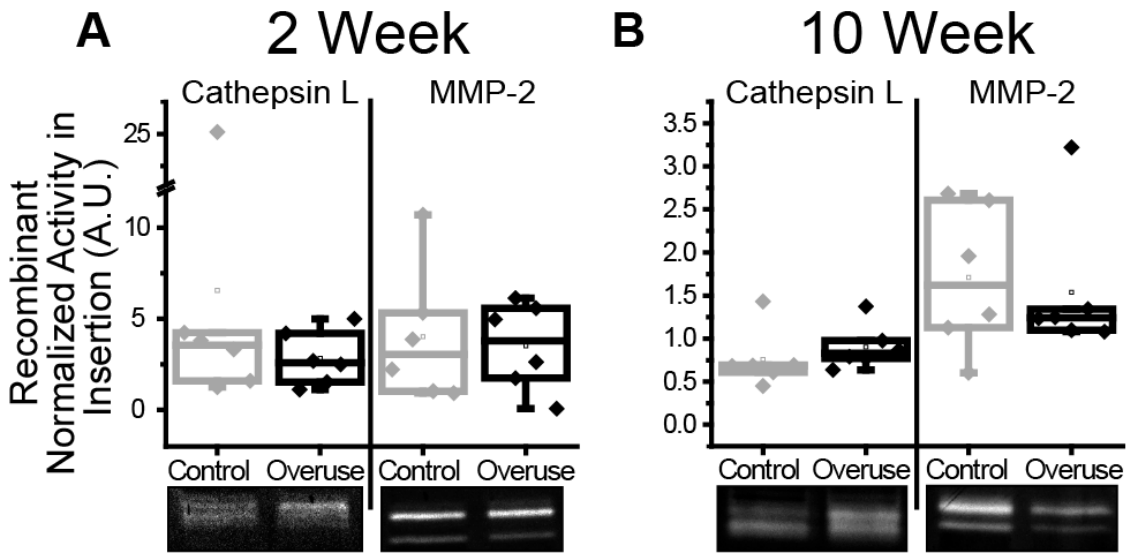

Figure 3.6. Cathepsin L and MMP-2 activity in supraspinatus tendon insertion. No significant differences in active rat cathepsin L or MMP-2 were measured by zymography between animals subjected to overuse or age-matched controls of rat supraspinatus tendon insertion after 2 weeks (A) (SD: ±9.2,1.5, 6.3, 3.7) or 10 weeks (B)(SD: ±0.3, 0.3, 0.8, 0.8) of overuse. Values were normalized to recombinant mouse cathepsin L and human MMP-2 in cathepsin and MMP gels, respectively. Zymography bands below all plots are representative of the corresponding protease, time, and condition (n=6).

3.3.6 Cathepsin and MMP activity in humeral articular cartilage

Cartilage tissue harvested from 10 week control and overuse rat humeral heads assayed by multiplex cathepsin zymography at pH4 exhibited unidentified active bands at 100 and 75kD (Fig 3.7A). MMP zymography on same tissues exhibited active pro- and mature MMP-2 bands at around 72 and 68kD, respectively (Fig 3.7C). Zymograms from both proteolytic families exhibited varying amounts of active proteases between individual animals, however neither cathepsin nor MMP activity demonstrated any significant difference between the control and overuse animals as quantified by densitometry (Fig 3.7B,D).

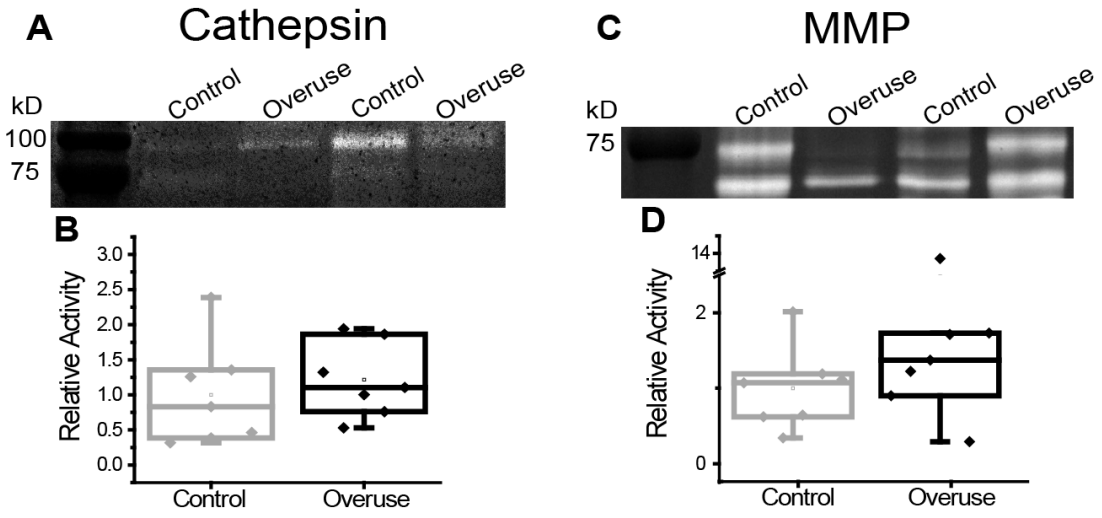

Figure 3.7. Cathepsin and MMP activity in humeral cartilage. Cathepsins appeared active in humeral head cartilage as visualized by zymography, with bands at 100 and 75 kD (A), but neither band demonstrated significant differences between control and overuse cartilage tissues (B) (SD: ±0.7, 0.5). MMPs also appeared active by zymography, with bands around 75 and 65 kD, indicative of the pro-form and mature form of MMP-2, respectively (C). However, neither band demonstrated significance between the control and overuse cartilage tissues (D) (SD: ±0.5, 4.7) (n=7).

3.4 Discussion

In addition to tendon degeneration, damage to glenohumeral articular cartilage can occur due to rotator cuff tendon injuries [95,96]. Studies have found decreased glenoid cartilage thickness, decreased compressive modulus, and diminished histological properties after full supraspinatus and infraspinatus rotator cuff tears [85,86]. However, to our knowledge, no studies have investigated the effect of overuse injury alone on the surrounding tissues. It has been shown that cytokines and proteases are upregulated in the synovial fluid after tendon tears, and that these synovial factors are positively correlated with the development of early osteoarthritic degeneration [86,97]. This suggests that proteases active due to supraspinatus overuse could be contributing to cartilage degeneration via the synovial fluid. Given this information coupled with our previous observation that damage occurred only at the tendon insertion region, we hypothesized concomitant degenerative effects of supraspinatus tendon overuse on tendon and humeral articular cartilage at 10 weeks.

Supporting this hypothesis, results demonstrated structural changes in rat supraspinatus tendon insertion and humeral articular cartilage after 10 weeks of supraspinatus tendon overuse. Histological scoring at the 2 week time point indicated significant variation in regional cellularity in the control supraspinatus insertions compared to the overused tissue (Table 3.1 and Fig 3.2A). Although unexpected, this may be a result of the natural level of variation in locations of cells within the tissues of both the control and overuse groups at 2 weeks. Furthermore, no other category showed any significant differences at this time point, indicating no overall histological evidence of pathology over 2 weeks. At 10 weeks, however, cellular shape and fiber organization, two of the main histopathological changes found in injured tendon [1] received

significantly greater scores in the overuse samples (Table 3.1 and Fig 3.2D), which is reflective of the changes in regional cellularity and shape previously observed after 4 and 8 weeks of overuse [5]. Taken together, these data suggest that tendon tissue damage becomes histologically evident at 4 weeks, continues to degenerate by 8 weeks, and persists at 10 weeks, which is consistent with findings from other studies [5,47,90]. Mechanical testing demonstrated no difference in insertion tensile modulus (Fig 3.2E) or overall tendon modulus (data not shown) between overuse and control animals, indicating that not enough damage has occurred to significantly change the overall modulus of the entire insertion region. However, the tendon insertion histological scoring and the observed humeral cartilage degeneration (discussed below) suggest that damage to the joint overall occurs as a result of the overuse injury.

In addition to quantifying fibrillar and cellular changes with H&E staining and scoring, second harmonic generation (SHG) was used to indicate changes in collagen fiber density as well as changes in characteristic collagen crimping patterns. Second harmonic generation is a non-linear microscopy technique that provides high resolution images samples of interest. Collagen is a noncentrosymmetric molecule, and when excited with a high intensity laser, the light waves interact, generating a second harmonic wave, or light that has twice the energy—twice the frequency and half the wavelength—of the original beam. Collecting these second harmonic waves and filtering out the others allows collagen-specific imaging that provides structural information across the tissue at high resolutions without the need of pinholes or exogenous labeling [55,92]. Collagen fiber crimping orientation and structural changes from arterial and bone tissue have previously been characterized using SHG [55]. Here, evidence of collagen crimp

disturbance and decreased collagen fiber organization in the overuse tendons (Fig 3.3) provided additional evidence of damage induced by overuse and proteolytic degradation without the need for staining.

To assess damage to articular cartilage in early stages of glenohumeral joint injury, we employed contrast-enhanced μCT (EPIC-μCT) [61,85,89]. The decreases observed in joint space distance, cartilage thickness, proteoglycan content, and increased subchondral bone thickness (Fig 3.4 & Fig 3.5) are all indications of osteoarthritic-like changes in the humeral head cartilage [59,89]. The articular cartilage degeneration observed may result from altered mechanical loading of the glenohumeral joint and/or proteolytic imbalances in the synovial fluid [45,59,85,98,99]. Such results are similar to those found in other rat models of traumatic shoulder injury involving tendon transection. In one study, cartilage thinning and a significant decrease in elastic modulus was observed at several regions across the glenoid in rats 4 weeks after supraspinatus and infraspinatus transection compared to their contralateral tissues [85]. Another study used a modified Mankin scoring system to quantify damage in glenoid cartilage tissue sections 12 weeks after either a supraspinatus and infraspinatus tenotomy or a suprascapular nerve transection. Significant damage was found as indicated by cellularity, proteoglycan content, structure, and tidemark integrity in both injury models when compared to controls [86].

Increased protease activity has been found previously in damaged tendon and cartilage tissue. Cathepsin K protein expression was found upregulated in a rabbit model of flexor tendon injury [19] and mRNA levels were elevated in human calcific tendinopathy [20]. Cathepsin K was also upregulated in human osteoarthritic cartilage

[100] and in articular chondrocytes of an osteoarthritic mouse model [67]. MMPs have shown increased expression and proteolytic activity, both in human ruptured supraspinatus tendons as well as overuse tissues in a rat model after 2 and 4 weeks of activity [3,79]. Increased MMP-2, -9, and -13 activity as indicated by zymography and fluorescent enzymatic assays was found in human osteoarthritic chondrocytes [87]. In our study, in order to understand the proteolytic contribution to tendon damage progression observed in this model of overuse injury, we used multiplex gelatin zymography to quantify active proteases [83,101]. From cathepsin and MMP zymography on supraspinatus insertion tissues we observed no significant upregulation of active forms for either of the protease families between control and overuse animals for either 2 or 10 weeks of injury (Fig 3.6A,B). However, the tendon histology data present indications of degeneration by 10 weeks. Our previous findings showed upregulation of cathepsins K and L at 4 weeks and cathepsin L at 8 weeks overuse [5]. Along with current data, it suggests that the proteolytic activity of both cathepsins and MMPs may be subject to multiphasic temporal regulation over the course of overuse injury, and in this particular model, are likely upregulated before 10 weeks and induce damage to the ECM as observed histologically as early as 4 weeks in our previous study [5].

The cartilage tissue cathepsin zymography presented high molecular weight active bands at 75 and 100kD (Fig 3.7). These bands are thought to be caused by cathepsins bound to extracellular matrix, as they were seen in supraspinatus tendon tissue and endometriosis lesions [5,102]. These cathepsins are likely bound to insoluble collagen and proteoglycans, and may become proteolytically active upon release from resident cells [25,73]. These data could provide a mechanism of collagen cleavage in a

tendon overuse context for future studies. Although neither cathepsins nor MMPs were upregulated in cartilage by 10 weeks of injury, it is possible that, similar to the tendon insertion, proteolytic upregulation in the humeral articular cartilage would occur before the 10 week point. Further work is required to investigate the time course of cartilage injury due to tendon overuse to better identify when therapeutics might be best introduced to reduce damage to both tissues.

This study included several limitations. The rat was chosen as a model of overuse due to coracoacromial arch similarities to the human shoulder. However, it is important to note that because rats are quadruped animals, their shoulders are not loaded in the same manner as in humans, and therefore do not completely recapitulate the mode of clinical overuse. While no known human studies are available that examine cartilage changes during tendon overuse, this study may motivate collection of more clinical data in this area. This study was also unable to distinguish whether cartilage or tendon degeneration initiates damage to other tissue in the joint, or if these occur concurrently.

In summary, this work has exhibited degeneration in multiple tissues adjacent to the humeral head as a result of an overuse protocol, as quantified by histological scoring of rat supraspinatus tendon tissues as well as cartilage thickness and dye attenuation as measured by EPIC-μCT. In tendon tissues, active cathepsin L and MMP-2 were not upregulated in overused tissues compared to their age-matched controls. Similarly, after 10 weeks of tendon overuse, humeral articular cartilage also showed no significant difference between control and overuse tissues in terms of proteolytic activity. This work suggests a necessity to treat both the tendon and nearby cartilage to slow or reverse tissue damage during glenohumeral overuse injuries. Taken together with our previous data that

indicates upregulation in cathepsins in tendon overuse injury, these results also highlight the importance of elucidating temporal regulation of proteolytic activity to best determine timing of any future therapeutic strategies employing protease inhibitors.

CHAPTER 4. Sequence Matters: Priming Type I Collagen-Rich Extracellular Matrix with Cathepsin K Before Incubating with Mouse Cathepsin L Results in Enhanced Degradation

This chapter was adapted from Parks et al. (2018). Sequence Matters: Priming Type I Collagen-Rich Extracellular Matrix with Cathepsin K Before Incubating with Mouse Cathepsin L Results in Enhanced Degradation. In preparation.

4.1 Introduction

Tendinopathy, also known as tendon overuse, is the most common tendon injury and can cause significant pain and limited range of motion. One of the clinical hallmarks of tendinopathy is the degeneration of tendon extracellular matrix (ECM) [1,46,52]. If left untreated, early tendon injury can progress to full tendon rupture, concomitant degeneration of other joint tissues, and abnormal joint mechanics [1,2,45,50,80,96]. Multiple etiological factors of tendinopathy contribute to pathological tendon degeneration, including mechanical overload that can cause microtrauma, and particularly dysregulated expression of proteases and their endogenous inhibitors in resident tenocytes [1,2,14,32]. Type I collagen accounts for 95% of the total collagen content in tendon with small amounts of types III and V [1,2,103–105]. Other tendon components include approximately 2% elastin and 1-2% proteoglycans, largely decorin and byglycan [1,2,45,105,106].

Collagenolytic proteases are important to tendon matrix homeostasis by facilitating normal collagen turnover and ECM remodeling during wound healing [2,3,66]. However, upregulated collagenases, namely cysteine cathepsins and matrix metalloproteinases (MMP), have been reported in tendinopathic progression

[2,3,5,32,40,61,63,66,79]. Cysteine cathepsins are a family of proteases, implicated in numerous tissue destructive diseases, including orthopaedic tissues [6,7,11–13,21]. CatK is the most potent mammalian collagenase, able to cleave the collagen molecule at multiple sites within the triple helix and at the telopeptide ends [21]. CatK is primarily responsible for normal osteoclastic bone resorption in both humans and mice [21,22], however, elevated catK mRNA levels were also measured in human calcified tendon tissues [20]. Increased catK expression was also found in a rabbit model of flexor tendon injury [19]. Human cathepsin V (catV) is the most potent mammalian elastase with some collagenolytic activity, and is orthologous to murine cathepsin L (catL). CatL is ubiquitously expressed in mice and was found to be upregulated in diabetes and cardiovascular diseases [8,23–25].

We have recently demonstrated upregulated active cathepsins in a downhill treadmill rat model of early stage rotator cuff tendon injury [5,80]. In our rat model, we observed an upregulation of both catK and catL after 4 weeks of overuse, and catL remained upregulated after 8 weeks of overuse, with evidence of tissue damage to tendon and articular cartilage by 10 weeks [5,80]. These data showed that the resident tendon cells produced and secreted multiple cathepsins at different stages of the overuse injury resulting in degradation of the tendon ECM, suggesting that cathepsin upregulation was important to the eventual tissue degeneration. Thus, elucidating how catK and catL work together to degrade tendon is imperative for the development of appropriate and effective therapeutics.

Some studies have investigated how multiple proteases, within and between families, work synergistically to degrade a shared substrate. One study showed that

cathepsins L, B, S, and F were only able to fully degrade type I collagen fragments in the presence of chondroitin-4 sulfate after first being cleaved by MMP-1 [25]. Increased degradation of type I collagen fibers with age-related modifications by catK have also reported after the fibers were initial cleaved by collagenolytic MMP-1, MMP-8, and MMP-13 [107]. Additionally, concurrent and sequential combinations of cathepsins B, L, D and calpain demonstrated varied synergistic capacities to degrade and disassemble myofibrillar protein of grass carp, suggesting that multiple cysteine proteases in sequence may be driving increased rate of tissue degradation [108]. Previously, it has been published that catK is subject to autodigestion, or self-cleavage over time [69]. Additionally, we have published that cathepsins can hydrolyze other cathepsins, even in the presence of a substrate, a phenomenon we have termed cathepsin cannibalism [69,109]. These studies suggest that cooperative substrate cleavage, autodigestion, and cannibalism between multiple proteolytic species can dictate the amount of active proteases in a system as well as the amount of tissue degradation. However, it is still unclear how multiple simultaneously present cathepsins impact tendon ECM degradation, particularly of type I collagen.

In this study we employed polymerized collagen gel and homogenized tendon ECM incubation models as test beds to investigate how catK and catL act in concert to degrade these collagen matrix substrates. These experimental systems were used to test the hypothesis that catK initiates substrate degradation, exposing sites susceptible to further hydrolysis by catL, and thus, pre-incubating matrix substrate with catK before introducing catL would prime the collagen molecules, resulting in greater hydrolysis than when concurrent co-incubated with catK and catL.

4.2 Methods

4.2.1 Collagen Gels Preparation

For collagen gel preparation, soluble rat tail type I collagen (Gibco/Thermo, Waltham, MA) at 3mg/mL was combined with deionized water, 10X phosphate-buffered saline (PBS), and 1N NaOH to obtain a 2mg/mL solution at pH between 6.5 and 7.5. 50µL collagen gels were polymerized in 1.5mL microcentrifuge tubes. After polymerization, gels were washed in a 0.1M sodium phosphate buffer with 2mM dithiothreitol (DTT) and 1mM ethylenediaminetetraacetic acid (EDTA) at pH6, hereafter referred to as pH6 assay buffer.

4.2.2 Mouse Achilles Tendon Isolation and ECM preparation

All animal procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Mice deficient in apolipoprotein E ($ApoE^{-/-}$) were obtained from Jackson Laboratory (Bar Harbor, ME). These animals were chosen because they share the background genotype with additional cathepsin deficient animal models for use in future studies. $ApoE^{-/-}$ mice were bred and progeny were raised to 2-4 months of age and then sacrificed. The left and right Achilles tendons were extracted from 37 $ApoE^{-/-}$ mice. Each tendon was separated from the gastrocnemius muscle tissue and bluntly cut at the calcaneous insertion point. Tendons were then frozen in PBS at -20°C until needed for homogenization.

For tendon ECM preparation, tendons from $ApoE^{-/-}$ mice were homogenized in zymography lysis buffer with 0.1mM leupeptin in tissue grinding microcentrifuge tubes with resin (Sigma-Aldrich, St. Louis, MO) and then centrifuged to remove the soluble tendon tissue lysate. The remaining homogenized tendon ECM was pooled, well mixed,

and redistributed into microcentrifuge tubes for each cathepsin incubation, including no-cathepsin controls. The insoluble tendon ECM was washed in pH6 assay buffer, and then the buffer was aspirated in preparation for incubations.

In preparation for the time course experiment, pooled insoluble tendon ECM was incubated in pH6 assay buffer for 8hrs at 37°C with slight agitation. Tendon samples were centrifuged and the soluble releasate (pH6 assay buffer with non-enzymatically released soluble tendon ECM fragments) was collected and pooled for subsequent cathepsin incubations.

4.2.3 Cathepsin incubations with collagen gels and tendon ECM

For all cathepsin incubations, recombinant human cathepsin K (ENZO, Farmingdale, NY) and recombinant mouse cathepsin L (Novoprotein, Summit, NJ,) were incubated at a 0.2μM concentration in pH6 assay buffer with collagen gels or tendon ECM, in addition to a no-cathepsin control with only pH6 assay buffer added to collagen gels or tendon ECM. All incubations were conducted at 37°C with slight agitation for 8 hrs total. Recombinant human cathepsin K (catK) and mouse cathepsin L (catL) were incubated individually with collagen gels or tendon ECM, with the same start point or concurrently, or in sequential combinations, where the primary cathepsin was pre-incubated alone for 4hrs and the secondary cathepsin was added at the 4hr mark continuing to 8hrs. For the sequential condition with collagen gels, gels were primed with catK and then incubated with subsequent catL (Fig 4.1).

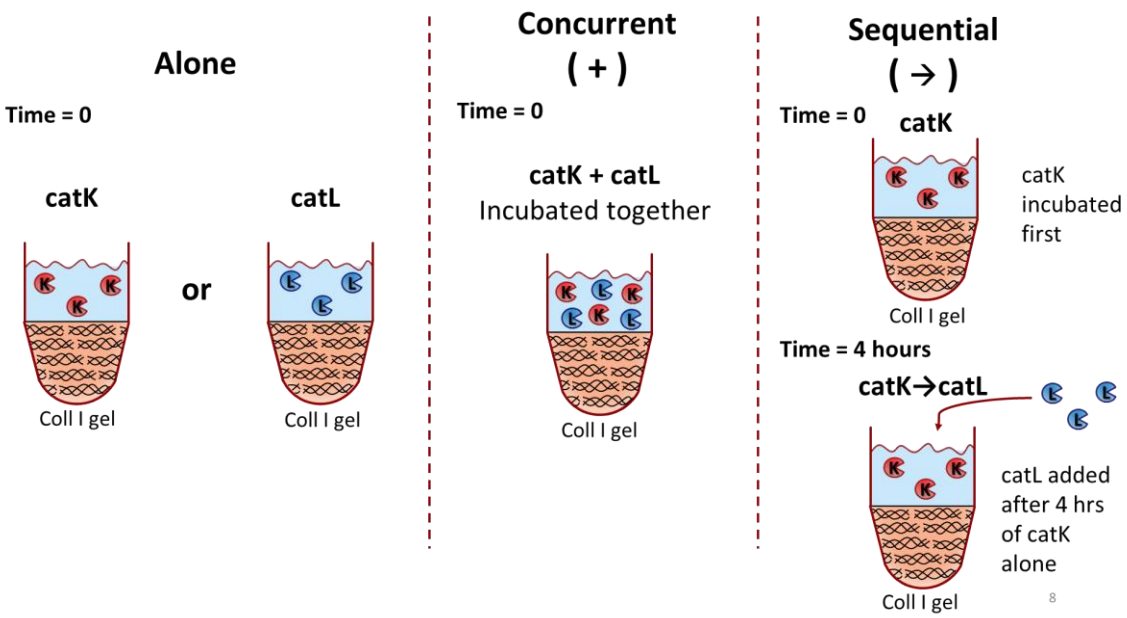

Figure 4.1. Schematic of catK and catL individual, concurrent, and sequential incubation conditions with type I collagen gels.

The sequential groups for tendon ECM were as follows: tendon ECM was 1) primed with catK and then incubated with catL, 2) primed with catL and then incubated with catK, 3) primed with catK and then incubated with additional, fresh catK, and 4) primed with catL and then incubated with additional, fresh catL. After the incubation period, to stop the reaction, leupeptin was added as a weak cathepsin inhibitor to each sample to a final concentration of 0.1mM. All samples were centrifuged at 13,200rpm for 15 min at 25°C. The supernatant was collected, referred to as the soluble fraction, and contained released collagen fragments and free, unbound cathepsins. The pellet fraction, containing the remaining insoluble collagen or tendon ECM along with any cathepsins associated, or bound to the matrix, was reconstituted with zymography lysis buffer with 0.1mM leupeptin.

In the time course incubation experiments, pooled soluble tendon ECM releasate was 1) incubated with concurrent catK and catL, 2) primed with catK for 4hrs before introducing catL, and 3) primed with catL for 4hrs before introducing catK. Each of these reactions was stopped after 4, 6, or 8hrs of incubation. In the two sequential conditions, for the 4hr time point, the reactions were stopped without the addition of the secondary cathepsin. At the end of each time point, leupeptin was added to each reaction to a final concentration of 0.1mM, and all samples were frozen at -80°C.

4.2.4 SDS-PAGE and Western Blotting

In preparation for electrophoresis, each soluble fraction and remaining pellet fraction were prepared with 5X reducing loading dye for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For Western blot, equal volumes of each sample were loaded into 12.5% polyacrylamide gels and separated by molecular weight. After separation, samples from each gel were transferred onto a nitrocellulose membrane. The remaining gels were stained with Coomassie Blue and destained, revealing bands indicative of type I collagen α1 and α2 chains, and β-chains, indicating an α-chain dimer [18,25,110–113]. Membranes were blocked with Odyssey blocking buffer, then washed in primary rabbit anti-human catK polyclonal antibody (Protein Tech, Rosemont, IL) overnight. The membranes were then incubated with anti-rabbit secondary antibody (LI-COR, Lincoln, NE) and imaged. Afterwards, membranes were stripped and re-probed with goat anti-mouse catL monoclonal antibody (R&D Systems, Minneapolis, MN) and appropriate anti-goat secondary antibody (LI-COR, Lincoln, NE) and imaged. All membranes were imaged using LI-COR Odyssey CLx (LI-COR, Lincoln, NE).

4.2.5 Multiplex cathepsin zymography

In preparation for electrophoresis, each soluble fraction and remaining pellet fraction were prepared with a 5X non-reducing loading dye for zymography. Zymography was performed as previously described [83,94]. Briefly, equal volumes of each sample were loaded into 12.5% SDS-PAGE gels embedded with a 0.2% gelatin substrate. After electrophoresis, gels were washed in renaturing buffer to allow the cathepsins to refold into their native, active confirmations. Then gels were incubated in pH4 sodium acetate or pH6 sodium phosphate buffers with 2mM DTT and 1mM EDTA for 18hrs at 37°C to allow the cathepsins to degrade the embedded gelatin substrate. After incubation, the gels were stained with Coomassie Blue and destained to reveal cleared white bands where active cathepsins degraded the substrate.

All gels were imaged with the ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, United Kingdom). Densitometry analysis was performed on the obtained images using ImageJ (NIH). SDS-PAGE collagen bands were normalized to the no-cathepsin control groups run in each gel. The cathepsin bands imaged in zymography gels and Western blots were normalized to 3.3 pmol of recombinant catK and catL run in each gel/blot.

4.2.6 Statistical Analysis

Statistical significance for SDS-PAGE, zymography, and Western blot data were determined by a one-way ANOVA in GraphPad Prism between each normalized incubation group with each replicate repeated at least three times and statistical significance considered when $p<0.05$.

4.3 Results

4.3.1 Sequential co-incubation of catK and catL cleaved more type I collagen gel and pooled tendon ECM than concurrent co-incubation

To test the hypothesis that priming matrix substrates with catK before the addition of catL would yield a greater amount of collagen cleaved compared to matrix co-incubated with catK and catL, collagen gels were incubated with either catK or catL alone, incubated with catK and catL added concurrently, or incubated with sequential catK and catL where the matrix substrates were primed with catK for 4 hrs prior to adding catL for the remaining 4hrs of the 8hr incubation period. After the incubation period, each sample was centrifuged and separated into the soluble (matrix fragment and free cathepsins) and pellet (insoluble matrix and bound cathepsins) fractions. Collagen cleavage in the pellet fractions and degradation in the soluble fractions was assessed with Coomassie stained SDS-PAGE. The amount of collagen cleaved is expressed as percent of collagen fragments present in the Coomassie stained polyacrylamide gel quantified with densitometry and normalized to collagen gel or tendon ECM in the absence of any proteases over the 8hr period.

Little to no collagen fragments were detected in the soluble fraction for any of the four conditions after incubation with the collagen gel. In the remaining collagen pellet fraction, α1(I), α2(I), and β-chains (α-chain dimers) were visible in all four conditions (Fig 4.2A). After normalizing to the no-cathepsin control, 93% of the collagen chains were cleaved in the sequential condition, significantly more than when catK or catL were incubated with collagen gels alone (29% and 37% cleaved, respectively) or co-incubated with collagen gels concurrently (55% cleaved) (Fig 4.2B).

Type I collagen gels were used initially as collagen is the major component in tendons, but to determine if the observations with collagen gels would be replicated on tendon matrix, a more complex mixture of ECM proteins, homogenized mouse Achilles tendon ECM was incubated with catK and catL in the same manner as the collagen gels. SDS-PAGE was used to assess the amount of collagen cleaved and degraded from the individual tendon ECM incubation method. Soluble and tendon pellet fractions were obtained after 8hr incubations with catK and catL. In the soluble tendon ECM control group that was incubated only with assay buffer, pH 6, and no cathepsins, there were many soluble tendon ECM fragments were present at multiple molecular sizes (Fig 4.2C,F), due to the partial solubility of collagen in the pH6 assay buffer [114]. When comparing catK alone, catL alone, concurrent co-incubation of catK and catL, and the sequential co-incubation of primary catK and subsequent incubation with catL, the soluble fraction showed that the soluble collagen remained only when incubated with catL alone. Significantly less collagen was measured in the soluble fraction of all other conditions when compared to the catL alone incubation (Fig 4.2C,D). The collagen in the tendon ECM pellet fraction showed no significant differences in the amount of collagen cleaved between any of the conditions, even when comparing concurrent co-incubation of catK and catL to the sequential co-incubation (Fig 4.2C,E).

SDS-PAGE showed that after separating control tendon ECM that had only incubated with pH6 assay buffer, the tendon pellet control had similar α- and β-chains visible as with the collagen gels. In the soluble tendon control group many soluble tendon ECM fragments are present at multiple molecular sizes, due to the partial solubility of collagen in the pH6 assay buffer [114]. In the experimental groups SDS-PAGE revealed

the most matrix was cleaved from the pellet fraction of tendon ECM primed by catK then incubated with catL, with 53% collagen cleavage (Fig 4.2F,H). For the soluble fraction, incubation with catK degraded significantly more collagen than in the absence of catK, regardless of priming, concurrent, or catK alone. When catL was incubated alone with tendon ECM, there was no degradation of the soluble ECM fragments (Fig 4.2F,G).

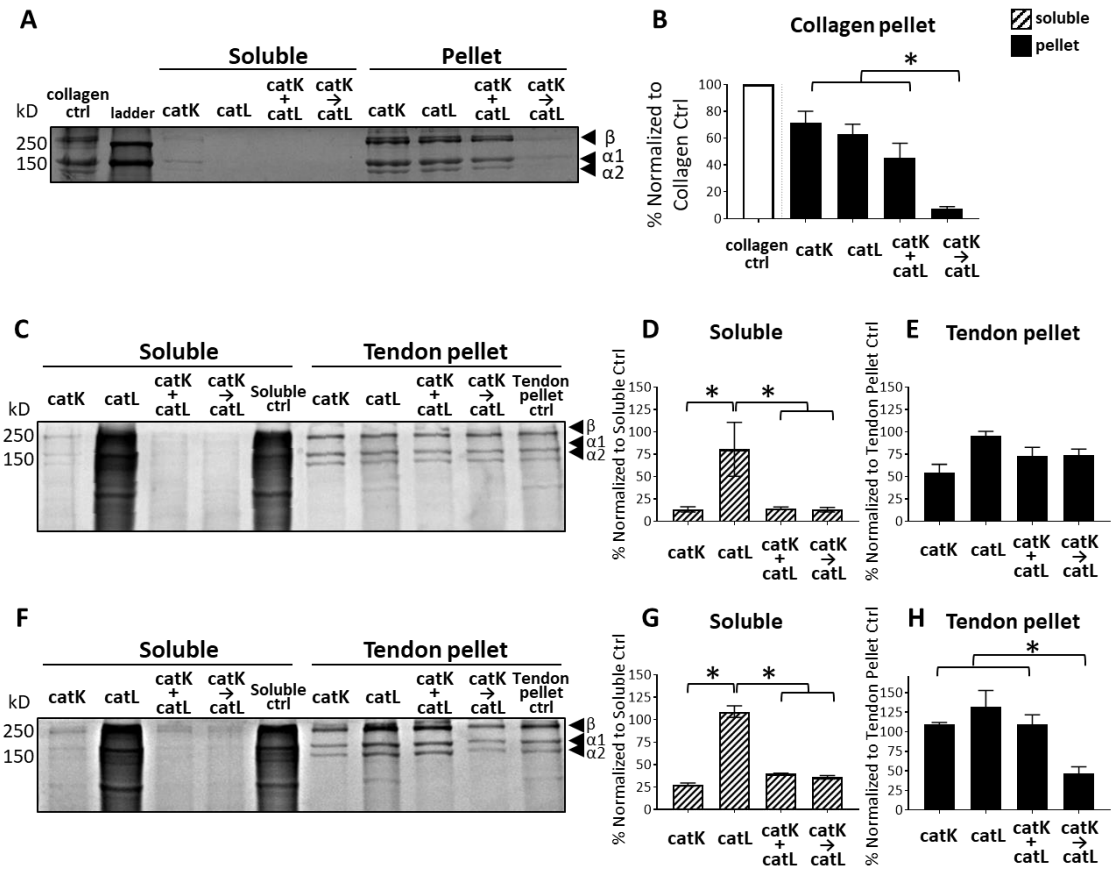

Figure 4.2. Matrix substrate co-incubated with sequential catK and catL yields greater collagen cleavage than concurrent catK and catL. Collagen gels and tendon ECM were incubated with catK alone, catL alone, and catK co-incubated with catL concurrently (+), or sequentially (→) for 8hrs. After centrifugation, SDS-PAGE was conducted on the soluble and pellet fractions of each experiment. SDS-PAGE showed the alpha chains from the collagen pellet with little to no collagen remaining in the soluble fraction after 8hr cathepsin incubations (A). Sequentially co-incubated catK and catL (→) cleaved significantly more collagen than catK concurrently co-incubated with catL (+), or either enzyme alone (B). SDS-PAGE on the soluble and pellet fractions of individually incubated and pooled tendon ECM showed the fragments released into the soluble fraction, and the remaining alpha chains in the pellet after incubation (C,F). In the soluble fraction of the individual and pooled tendon incubations, catL alone was not able to degrade the ECM fragments, leaving significantly more fragments than catK alone, concurrent (+) and sequential (→) catK and catL (C,D,F,G). The pellet fraction of the individual incubations showed no differences in the amount of cleaved tendon ECM between groups (C,E). In the pooled tendon ECM pellet fraction, similarly to the collagen pellet, SDSPAGE showed significantly greater cleavage with sequential co-incubation (→) than catK alone, catL alone, or concurrent co-incubation of catK and catL (+) (F,H). n=3, * indicates statistical significance, $p<0.05$ as determined by a one-way ANOVA.

4.3.2 Cathepsins remained present and active, and displayed cannibalistic behaviors after 8hr incubations with collagen gels

After observing that catK priming collagen I, followed by catL cleavage, yielded significantly higher degradation of type I collagen and tendon ECM from the pellet fraction than when they were concurrently co-incubated, we next investigated functional dynamics between catK and catL that could cause this difference. Active cathepsins are subject to autodigestion, or self-cleavage over time, cathepsin-on-cathepsin hydrolysis, a condition we termed cathepsin cannibalism, and other mechanisms that could inactivate without cleavage [69]; any of these processes could reduce the amount of active cathepsins present in the system, reducing total substrate degradation. We tested the hypothesis that catK or catL could be cannibalizing one another in the concurrent co-incubation condition, reducing matrix degradation compared to when catK was pre-incubated with the matrix prior to addition of catL. To measure the amount of active cathepsins remaining after the matrix substrate incubation period, zymography was performed on the soluble and reconstituted collagen pellet fractions. Zymography showed active catK remained after the 8hr incubation period in both the pellet and soluble fractions (Fig 4.3A), however, there was significantly higher amounts of active catK remaining when it was incubated alone, in the absence of catL, not added concurrently or sequentially. These data suggest that in the presence of the collagen gel, the catK was stabilized, and protected from autodigestion; this was confirmed by Western blot for catK protein (Fig 4.3D). There was a significant loss of catK protein when incubated with catL, either concurrently or sequentially, suggesting cannibalistic interactions of catL on catK, but the loss of catK was significantly reduced in the sequential case compared to the concurrent co-incubation condition (Fig 4.3D,E).

Zymography also showed a loss in the amount of active catL in the collagen pellet fraction when co-incubated with catK either concurrently or sequentially (Fig 4.3A,C). This was confirmed by significantly more immunodetectable catL protein when incubated alone than when either concurrently or sequentially co-incubated with catK on the collagen gels (Fig 4.3F). Together these results suggest bidirectional cathepsin cannibalism, with catK hydrolyzing catL and catL hydrolyzing catK. There was more catK in the pellet fraction than in the soluble fraction, but the opposite for catL with a greater amount of catL in the soluble fraction.

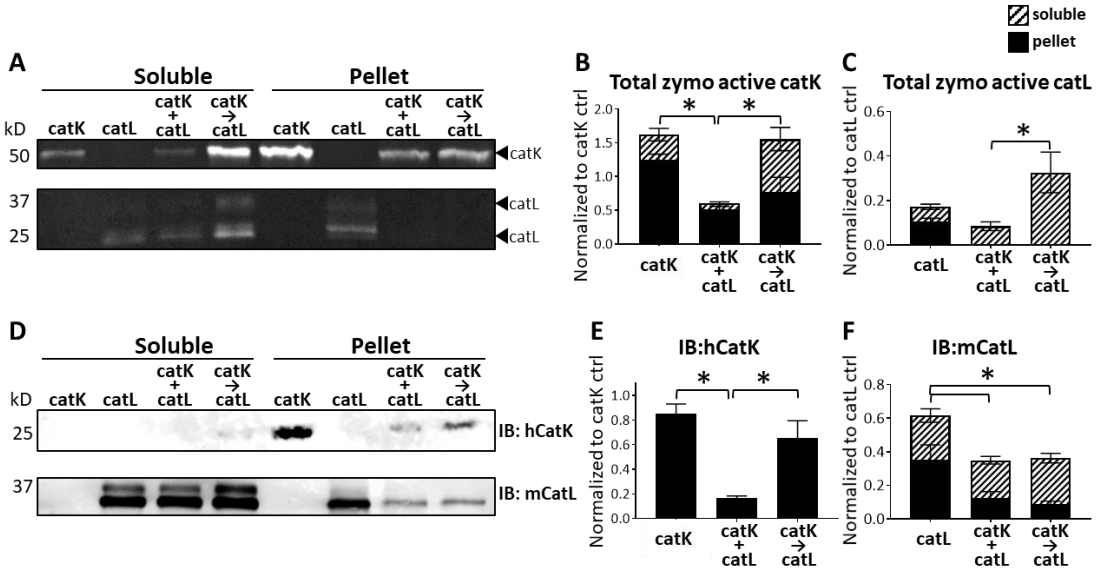

Figure 4.3. Sequential co-incubation of catK and catL with matrix substrate sustained active catK, while bi-directional cannibalism of catK and catL occurred with concurrent co-incubation. Zymography and Western blots against catK and catL were conducted to measure the amount of active catK and catL remaining after incubation with collagen gels and tendon ECM. CatK and catL were active (A) after 8hr incubations with collagen as indicated by zymography in the soluble and pellet fractions. Significantly more total catK was active when collagen is sequentially co-incubated catK and catL (→) than the concurrent co-incubation (+) (B). More total catL was active when collagen is primed with catK (→) than the concurrent co-incubation with catK (+), due to the reduced incubation time (C). CatK Western blot detected no catK in the soluble fraction, but also showed in the pellet fraction less total catK when concurrently co-incubated with catL (+), indicating catL cannibalism of catK (D,E). By Western, less total catL was present when co-incubated with catK, even when sequentially co-incubated with catK (→), suggesting catK may also have been cannibalizing catL (D,F). n=3, * indicates statistical significance, $p<0.05$ as determined by a one-way ANOVA.

4.3.3 CatK cannibalized catL when co-incubated without substrate

Active catK was present in both the soluble and pellet fractions after incubating with collagen gels for 8hrs, suggesting that the presence of substrate increases the stability of proteases in the system, whereas it has been reported that with soluble substrates or simple amino acid short peptide substrates, they were inactivated over time periods of two hours [69]. To determine if substrate was influencing the dynamics during the co-incubation conditions, we next incubated catK and catL alone or together, in the absence of substrate. The hypothesis was that, according to enzyme kinetics, in the absence of substrate, the cathepsins would bind to and hydrolyze each other more than when substrate is present, as though additional substrates were competitive binders for the active cathepsins. When catK was incubated alone, catK autodigestion occurred as indicated by the loss of the active catK band over time in the zymogram, even in the absence of any other protease (Fig 4.4A,B). This was corroborated by the loss of catK protein over time in the immunoblots (Fig 4.4A,D). Some catL autodigestion occurred, but not to the same extent as catK (Fig 4.4). When both catK and catL were concurrently co-incubated, in the absence of substrate, there was a significant loss of active catL signal in the zymogram by 8hrs that was not seen when catL was incubated alone (Fig 4.4C).

To test if catL was still lost from the system after its sequential addition, catK was incubated alone for 4 hours, then catL was added for the remaining 4 hours to match the protocols of the collagen gel experiments from Figure 4.3. Whereas with collagen substrate, catK was detected after 8 hours in the soluble fraction after sequential addition of catL (Fig 4.3A), in the absence of substrate for this experiment, there was a significant loss of detectable active catK by zymography and total catK protein by immunoblot after

8hrs (Fig 4.4A, B). For shorter sequential co-incubation periods, with accompanying shorter catK pre-incubation periods of 0.5 and 2 hours prior to addition of catL, in the 1 and 4hr sequential conditions, respectively, a time dependent loss of catK was observed. CatL, on the other hand, showed no significant loss, similar to when it was incubated in the presence of collagen.

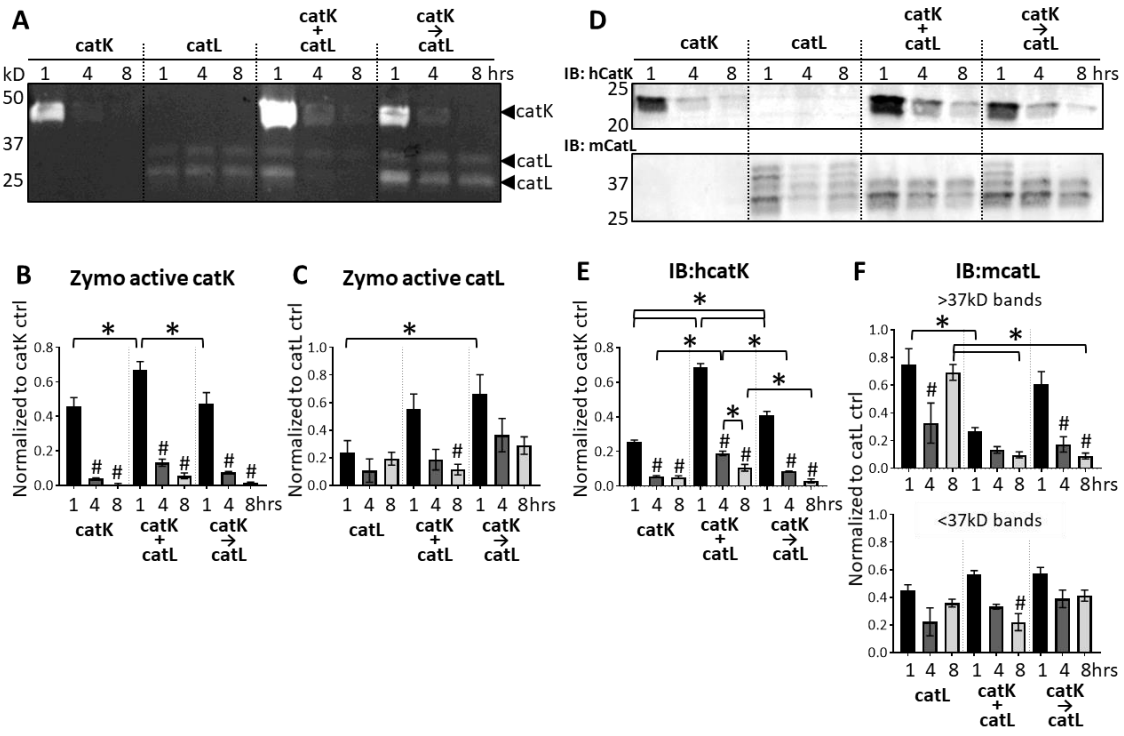

Figure 4.4. CatK cannibalized catL when co-incubated without substrate. CatK and catL were incubated without substrate for 1, 4, and 8hrs either alone, concurrently (+), or sequentially. In the sequential conditions, catL was introduced at the halfway point of each time point (0.5, 1, and 4hrs, respectively). Zymography showed active catK and catL remaining after incubation (A). CatK and catL are active at 1hr, with 4hr and 8hr time points showing decreasing signal, indicating auto-digestion (B,C). Significantly more catK was active when concurrently co-incubated with catL (+) compared to catK alone and sequential co-incubation of catK and catL (→) (B). However, active catL significantly decreased over time when co-incubated with catK (C). Confirming zymography trends, significantly more catK was detected by Western blot when co-incubated with catL compared to catK alone. Furthermore, significantly increased catK was detected with longer exposure to catL in the concurrent co-incubation compared to the sequential co-incubation at all time points (D). Western blot also showed catL significantly decreasing over time when co-incubated with catK, even after 1hr and even when catL was added sequentially, suggesting catK cannibalism of catL (E). n=3, * indicates statistical significance, # indicates statistical significance from the corresponding 1hr bar within the same condition, $p<0.05$ as determined by a one-way ANOVA.

4.3.4 Similar levels of tendon ECM cleavage were observed between tendon primed with catK before incubating with catL and tendon primed with catL before incubating with catK

Our central hypothesis of this study was that priming of collagen I by catK would cleave and expose additional sites susceptible to hydrolysis by catL, but the question remained of whether catL could prime for greater cleavage by catK or if subsequent addition of fresh protease of the same type to the system would have similar effects on matrix degradation as the mixed combination of catK/catL. To test this hypothesis, tendon ECM was incubated with the following catK/catL combinations: 1) primed with catK, then incubated with catL, 2) primed with catL, then incubated with catK, 3) primed with catK, then incubated with fresh catK, and 4) primed with catL, then incubated with fresh catL. Each priming/preincubation period was 4hrs, then the subsequent addition of the secondary protease for an additional 4 hrs as was done above.

For the soluble fraction, priming with catL and sequential co-incubation of fresh catL only degraded 23% ($n=3$, $p<0.05$) of the no protease control, but there was 85% degradation when catK was the secondary protease after priming with catL ($n=3$, $p<0.05$). Conversely, priming with catK, and subsequent incubation with catL led to 89% of the collagen being degraded, and 93% was degraded when the secondary protease was fresh catK (Fig 4.5A,B). This was significantly higher than either of the catL priming conditions ($n=3$, $p<0.05$). From the tendon ECM pellet fractions, 71% collagen was cleaved when primed with catK and then incubated with catL, significantly higher than the 26% cleaved from tendon ECM that was primed with catL, and then subsequently incubated with fresh catL (Fig 4.5A,C).

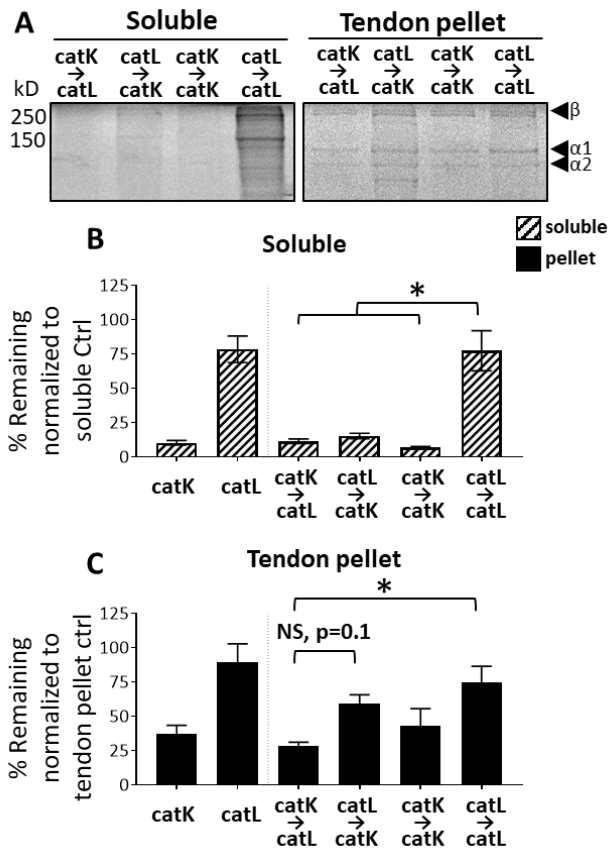

Figure 4.5. Tendon ECM primed with catK and then incubated with catL did not cleave more than tendon primed with catL and then incubated with catK. Tendon ECM degradation and active catK and catL were evaluated after 8hr sequential co-incubations with either catK or catL as the primary protease. SDS-PAGE showed that primary catK results in greater cleavage in the tendon ECM pellet (A,C) and greater degradation in the soluble fraction (A,B) than primary catL, though not significantly different. n=3, * indicates statistical significance, $p<0.05$ as determined by a one-way ANOVA.

4.3.5 CatK cannibalized catL when co-incubated in the presence of tendon ECM for 8hrs

It was hypothesized that catK and catL would be present and active after incubating with the tendon ECM substrate for 8hrs. Additionally, it was hypothesized that priming tendon ECM with catK would yield more active and total measured catK and catL signal than priming ECM with catL. To test these hypotheses, zymography was performed to assess for active catK and catL, and Western blots were probed for catK and catL to detect total protein amounts. We first confirmed that no resident cathepsins were active from the tendon ECM after homogenization by incubating the substrate in either pH6 assay buffer only or with exogenously added catK. Active catK signal was observed by zymography only when catK was exogenously incubated with the tendon ECM (Fig 4.6).

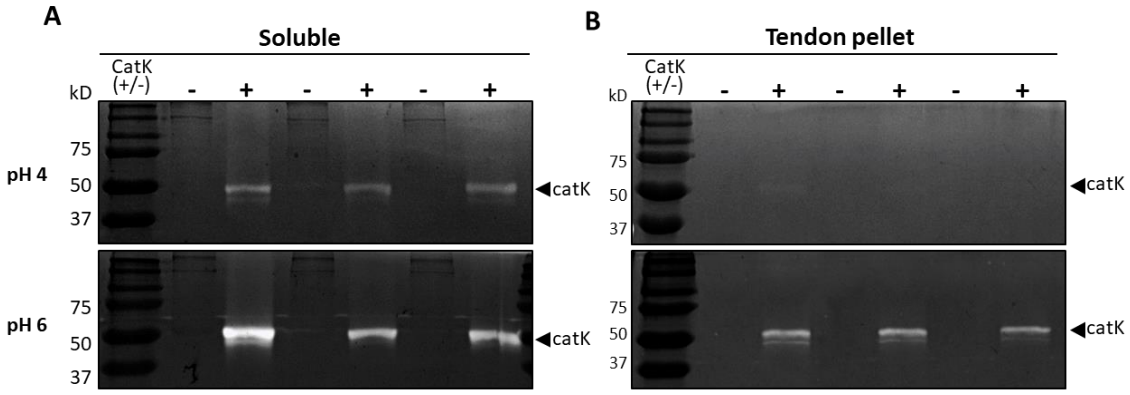

Figure 4.6. CatK retained activity in the presence of homogenized tendon ECM after 8hrs of incubation. Zymography shows active catK signal only when homogenized tendon ECM was exogenously incubated with catK, indicating no residually active cathepsins from the tendon ECM alone.

Zymography was performed to assess amounts of active catK and catL under these new co-incubation conditions, and Western blots were used to probe for catK and catL total protein, together to determine the extent and direction of cathepsin cannibalism. From the zymograms, there was little to no active catK and catL detected in the zymogram of the soluble fraction, but the amounts of active catK and catL in the pellet fractions was similar between each condition. This indicates that most of the active cathepsins remaining after the 8hr incubations with tendon ECM were associated with the tendon pellet and not free in the soluble fraction (Fig 4.7A).

Immunoblots probing for catK similarly revealed detectable catK only in the tendon pellet fraction after the 8hr incubations. No differences were measured in the total amount of catK present between any of the three conditions when catK was incubated with catL (Fig 4.7B, data not shown). CatL was detected by Western blot in both the soluble and tendon pellet fractions after incubating with tendon ECM for 8hrs. Interesting, only under conditions when catK was incubated with catL is there significantly less immunodetectable catL protein when compared to catL alone (Fig 4.7F,G).

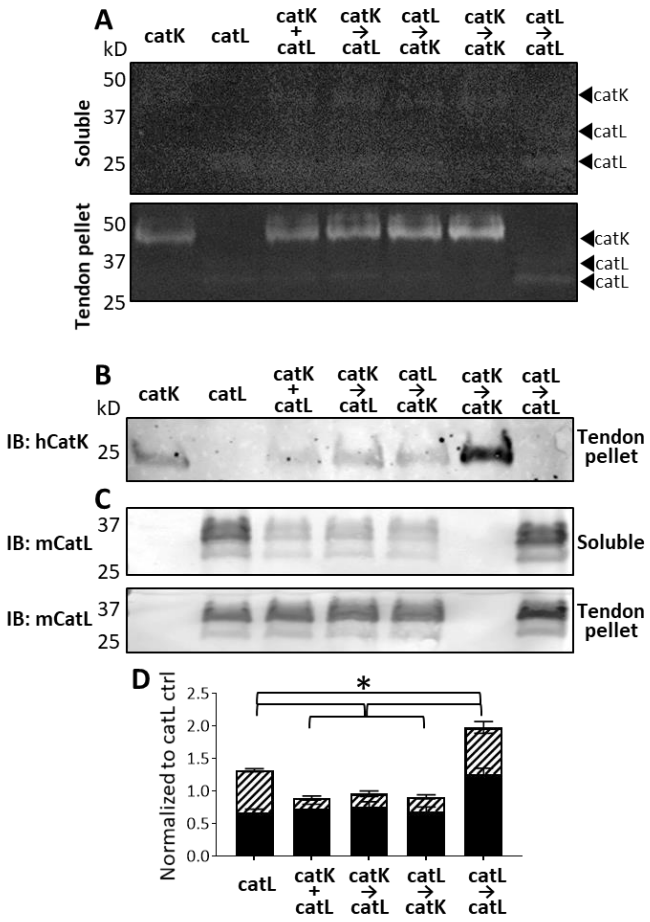

Figure 4.7. CatK cannibalized catL when co-incubated in the presence of tendon ECM for 8hrs. Zymography revealed limited active cathepsins in the soluble fraction, while similar amounts of active catK and catL remained associated with the tendon ECM pellet regardless of the priming sequence (A). Western blot for catK detected greater signal with tendon ECM primed with catK than tendon primed with catL, however, only tendon ECM primed and subsequently incubated with catK was significantly different from the other groups (B, data not shown). Western blot for catL showed similar levels detected in the pellet fraction, regardless of incubation conditions, but significantly less catL present in the soluble fraction when catL was co-incubated with catK (C,D). n=3, * indicates statistical significance, $p<0.05$ as determined by a one-way ANOVA.

4.3.6 Soluble tendon ECM was degraded into small fragments that stabilize cathepsins during time course incubations

The soluble fraction of tendon ECM was degraded significantly when incubated in the presence of catK. Beginning with insoluble ECM (collagen gels or tendon ECM pellet), our model assumed that catK and catL hydrolyzed protein in the bulk pellet, solubilizing it, and releasing it from the bulk into the supernatant. Any active protease in the supernatant could then bind and hydrolyze the released fragments or bind and hydrolyze more ECM protein in the bulk pellet. These solubilized protein substrates could distract the active cathepsins from further cleavage of the bulk pellet, while also stabilizing the active cathepsins in the soluble fraction that would now have substrate protein, which was demonstrated earlier to sustain the active cathepsins.

To test if the solubilized tendon fragments could stabilize the active catK and catL, extending their active time in the absence of bulk pellet ECM, and if cathepsin cannibalistic interactions during co-incubations or sequential incubations altered their levels, tendon ECM was incubated in assay buffer with no protease for 8hrs to collect non-enzymatically released soluble ECM fragments (soluble control, Fig 4.2C). These solubilized tendon ECM fragments were then concurrently co-incubated with catK and catL, primed with catK and then sequential incubation with catL, or primed with catL and then subsequent incubation with catK. The reactions were stopped at different time points to assess the loss of active enzyme and substrate over time: 1) at 4hr time point before addition of the secondary protease, 2) at the 6hr point, 2hrs of secondary protease co-incubation, and 3) at 8hr time point, 4hrs of secondary protease co-incubation. Control samples of soluble tendon ECM fragments were incubated for 4, 6, and 8hrs in the absence of any proteases.

Soluble tendon ECM fragments were degraded below the detection limits of the Coomassie stain by 4 hours when either primed with catK or when concurrently co-incubated with catK. Priming with catL did not degrade these fragments by 4 hours, but when sequentially incubated with catK, there was degradation during those 2 and 4 hours, but by the end of those incubation periods of 6 and 8hrs, respectively, soluble tendon ECM fragments were still visible (Fig 4.8A). Although no ECM fragments were detected in the stained polyacrylamide gel, their presence as a substrate were still sufficient to stabilize active catK and catL for the 6 and 8hr periods as shown by the zymography (Fig 4.8B) and by Western blots (Fig 4.8C), regardless of the co- or pre-incubation condition. Additionally, by zymography, active cathepsin bands are present around 250 kD, indicating active cathepsins were binding to larger ECM fragments as well as smaller generated fragments from their hydrolytic activity (Fig 4.8B).

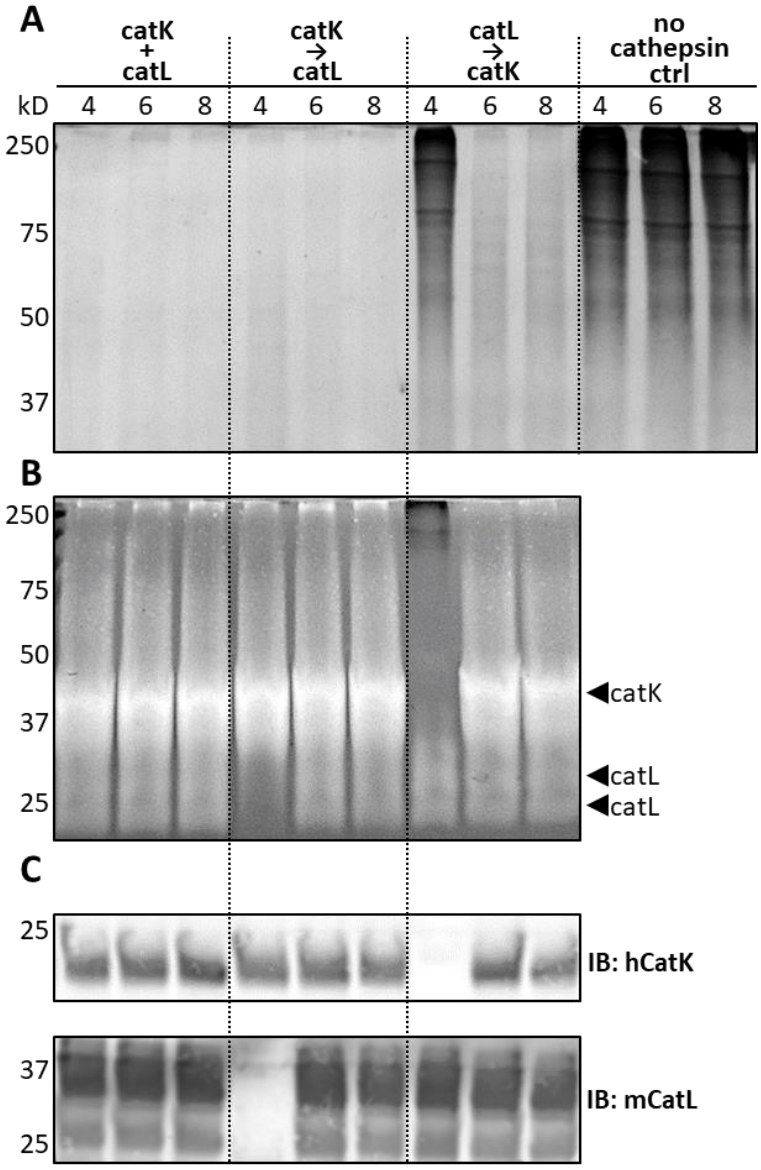

Figure 4.8. Co-incubation time course with soluble tendon ECM showed stabilization of catK and catL with greatest degradation when catK was initially present. Concurrent and sequential catK and catL were incubated with soluble tendon ECM for 4, 6, and 8hrs. SDS-PAGE showed the soluble tendon ECM remaining after incubation time course. At all time points with either the concurrent co-incubation or when soluble tendon ECM was primed with catK before the addition of catL, the ECM fragments were below Coomassie detection. Soluble tendon ECM primed with catL was degraded only after catK was added, with fragments still visible even by 8hrs (A). Soluble tendon ECM sustained active catK and catL by zymography at all 3 time points. CatK was active and associated with tendon ECM fragments at multiple molecular sizes throughout the zymography gel (B). Similarly, by Western blot catK and catL were detected at similar levels at all 3 time points where added (C). n=3.

4.4 Discussion

This study demonstrated that priming collagen matrix substrates with catK and then incubating with catL resulted in greater cleavage than when the substrates were concurrently co-incubated with catK and catL (Fig 4.2). Lower catK and catL signal was measured by zymography and Western with concurrent co-incubation of catK and catL with collagen gels when compared to the sequential co-incubation (Fig 4.3). This suggested that the limited matrix cleaved in the concurrent co-incubation condition could be due to cathepsin cannibalism. In the catK and catL incubations without substrate, we demonstrated that catK cannibalized catL, suggesting the presence of substrate impacts the hydrolytic behavior of catK (Fig 4.4). After identifying increased cleavage of tendon ECM when primed with catK and then incubated with catL, sequential groups were compared varying the cathepsin that primed the tendon ECM. It was found that when catK was co-incubated with catL, regardless of sequence, catK cannibalized catL. However, the sequential co-incubations of catK and catL did not differ in the amount of cleaved tendon ECM (Fig 4.5, 4.7). These data suggested that differences in tendon matrix cleavage happened at an earlier point during the incubation time course. Interestingly, soluble tendon ECM incubated with concurrent and sequential catK then catL degraded tendon fragments before sequential catL then catK. These fragments, even though below Coomassie detection limits still stabilized catK and catL by zymography and Western blot (Fig 4.8).

In order to understand how cathepsins cooperatively facilitate early tendon injury, the biochemical mechanisms that facilitate degeneration of tendon macromolecular structure need to be identified. Many studies have revealed how individual proteases are able to degrade extracellular matrix substrates [25,42,115–118]. However, it is known

that cathepsins are part of a complex proteolytic network with interacting species that enhance and inhibit proteolytic potential of multiple families [58,68,69]. Given the temporal upregulation of catK and catL in the previously rat rotator cuff tendon injury model [5], the objective of this study was to investigate how concurrent and sequential co-incubations of catK and catL act in concert to degrade a type I collagen-rich substrate. The hypothesis was that sequential catK then catL would result in greater substrate degradation than concurrent catK and catL. Specifically, we tested the hypothesis that catK, as the most potent mammalian collagenase, initiated collagen degradation by cleaving at multiple sites, exposes sites further susceptible for catL to continue cleavage.

Our finding of increased cleavage of matrix substrate from the pellet fraction of collagen gels and pooled tendon ECM when primed with catK and then incubated with catL suggest that introducing proteases to a substrate in sequence results in more efficacious matrix degradation. Priming matrix with catK allows cleavage of the matrix substrates, generating soluble fragments and freeing other cleavage sites, allowing for continued cleavage with the addition of catL. In an *in vivo* tendon context, the cleavage of the pellet fraction most closely reflects the potential degradation of tendon ECM in the presence of cathepsins. However, we found that when fragments were cleaved and released into the soluble fraction, even after further degradation, these fragments were able to stabilize active cathepsins for much longer than if the proteases were without substrate. This suggests that in the local tendon microenvironment, after insoluble matrix is cleaved, if not cleared away in synovial fluid, degraded fragments may be able to sustain active cathepsins for prolonged cleavage and degeneration, even if only small concentrations of cathepsins are present.

After incubating collagen gels with catK and catL for 8hrs, zymography and Western blot data suggested catK and catL may be cannibalizing each other when concurrently co-incubated with the collagen gels, distracting from degradation of the substrate. We have previously reported the propensity for cathepsin S to cannibalize catK even in the presence of an collagen substrate [69]. This is the first time we have demonstrated bi-directional cannibalism between catK and catL in the presence of a collagen substrate. Given that catK and catL were present and active after 8hr co-incubations with a collagen substrate, in addition to the cannibalistic behaviors observed between the two, we evaluated the effect of cathepsin co-incubation over time without substrate. From the observed results with the collagen substrate, the hypothesis was that the stability of catK and catL, and the dynamic interactions between the two would change over time. The amount of catK detected by zymography and western decreased by 8 hrs, due to autodigestion (Fig 4.4), unlike when in the presence of collagen. Contrary to the zymography results from the collagen gel incubations, more catK was detected by zymography and Western blot when co-incubated with catL than when incubated alone (Fig 4.4B,D). Unlike catK, catL was particularly stable over time when incubated alone. However, less catL was immunodetectable over time when co-incubated with catK (Fig 4.4E), all suggesting that without substrate catK cannibalizes catL and this cannibalistic action protects catK from autodigestion observed when catK is incubated alone. These data point to a single direction of cannibalism when no substrate is present. However, when in the presence of collagen, catK and catL display bi-directional cannibalism, suggesting that the substrate does dictate some of the proteolytic dynamics, yielding substrate-dependent responses.

We compared multiple sequential incubation conditions to test the hypothesis that tendon ECM primed with catK and then incubated with catL degrades more ECM than when tendon was primed with catL and then incubated with catK. Most of the fragment cleavage and degradation occurred in the sequential co-incubations primed with catK, though the quantification did not show significant differences. The presence of multiple types of macromolecules in the tendon ECM besides collagen, such as elastin and proteoglycans, could have contributed to the confounding quantifications [40–42,119]. The comparison between the sequential conditions yielded more subtle differences and could be a result of the complex interactions between the cathepsins and the varying components of the tendon ECM milieu. Studies have published on the modifications in cathepsin activity by glycosaminoglycans, particularly in acidic conditions [25,35,73]. CatL Western blot revealed no significant differences between concurrent and sequential co-incubations of catK and catL, but catL—both alone and sequentially as primary and secondary cathepsins—was different from all other groups. Similarly to the collagen gel incubations, these results suggest cannibalism of catL by catK with co-incubation, even when catL is the primary cathepsin and has an opportunity to bind to matrix substrate.

To differentiate between the amount of soluble tendon ECM degraded and active catK and catL over time between concurrently and sequentially co-incubated with catK and catL, we conducted a time course experiment, incubating each group and stopping the incubations after 4, 6, or 8hrs. Though it appeared by SDS-PAGE that fragments were fully degraded in the concurrent incubation and when soluble tendon ECM was primed with catK, active catK and catL were both present at all three time points in all three co-incubation conditions where added. Additionally, catK appeared active

continuously through the zymography gel suggesting that catK was associated with fragments at many different molecular sizes. In the 4hr sequential condition with catL, where catK was not added, this pattern is not observed, suggesting catL did not associate with these fragments. We and others have published that cathepsins are active by zymography while still associated with substrate [5,80,102,120,121]. This result contrasted with the zymography results from the direct incubations with the tendon ECM, where very little active catK and catL were observed in the soluble fraction. This difference in cathepsin behavior may suggest that catK and catL have a higher affinity for larger tendon ECM macromolecules, but will still bind to and hydrolyze smaller, more soluble fragments. Furthermore, studies have shown that certain GAGs, in addition to serving as a substrate for cathepsins, enhance cathepsin K activity [14,25,34]. Particularly as there were no larger insoluble macromolecules to bind to in the time course experiment, the soluble substrate incubations served as substrate for binding and stabilizing active catK and catL over the 8hr time course. Overall, this data set suggests that catK and catL may prefer larger insoluble tendon matrix macromolecules, but that even the presence of soluble tendon ECM fragments stabilizes active catK and catL and serves as a reservoir for extended hydrolysis.

The findings of this study have many implications for understanding how cathepsins contribute to the progression of tissue destructive diseases characterized by matrix degradation. Intra-and interfamilial proteolytic crosstalk, not just the actions of a single protease, may be driving degeneration [5,80,108]. These data provide motivation to study cellular mechanisms of proteolytic upregulation. Specifically in tendon disease, cathepsins K and L [5,80], MMP-1, -2, and -13, and their endogenous inhibitors, are

dysregulated in rat and human tendinopathy [1,3,63,79]. Further studies on the interactive effects of cathepsins and MMPs on tendon degeneration are necessary to determine an appropriate strategy for preventing disease progression. Cathepsins are very tightly regulated proteases for deliberate substrate degradation, but can display substrate promiscuity [11,13,14,16]. The stabilization of active catK by the presence of either a collagen substrate or catL, in a way that is not observed when incubated alone, suggests that even brief upregulation of secreted and active catK in a tendon injury context can result in substantial matrix degeneration.

Although difficult to identify *in vivo,* the findings here on cannibalistic behaviors of catK and catL, particularly in the presence of a collagen substrate, point to potential regulatory efforts by tenocytes to restrict potent collagenases from excessive matrix hydrolysis. This could imply that only one of a series of upregulated cathepsins would be the ideal target of inhibition. Alternatively, it could imply that inhibiting only one protease at one time, or even one family of proteases, may be insufficient to arrest the progression of disease. Cannibalism may also prove a useful strategy for localized cathepsin inhibition. Currently all available cathepsin inhibitors have failed phase II and III clinical trials due to unintended side effects [9,122,123]. We also have shown that direct cathepsin inhibition may inhibit certain cathepsins, but result in counterintuitive upregulation of others [70]. Because we know the proteolytic network is connected [58], introduction of inhibitors specific to cathepsins and/or MMPs may have unintended consequences for tendon tissue homeostasis, and thus this impact will have to be elucidated.

This study presented with several limitations. Mouse catL was used in the incubation experiments because catK and catL were found upregulated in our published model of early stage rotator cuff injury in rats. However, the murine catL ortholog, human cathepsin V, would provide more clinical relevance in future translational studies investigating cathepsin-mediated tendon matrix degeneration. Furthermore, human catK was also used in these studies because there is no commercially available murine catK. Although the catK between these two species display pointed differences in response to certain inhibitors [77], catK is also the only cathepsin unambiguously implicated in the bone resorption in both humans and mice—a process dominated by the degradation of type I collagen [22]. This study used homogenized tendon ECM as a test bed for degradation and active enzyme levels in co-incubations. This system does not completely recapitulate proteolytic actions on fully organized tendon, but helped to eliminate any confounding proteolytic impact from intracellular proteases present in resident tenocytes.

In summary, we have demonstrated increased hydrolysis of collagen-based matrix substrate with sequential co-incubation of catK and catL when compared to concurrent co-incubation of catK and catL, with evidence of enzyme stabilization even through 8hrs. This study has also revealed substrate dependent cannibalistic behaviors between catK and catL with implications for the progression of tendon degeneration. We also showed that even though priming tendon ECM with catK and then incubating with catL did not hydrolyze more substrate than then inverse by the end of 8hrs, priming with catK resulted in hydrolysis of soluble tendon ECM fragment below Coomassie detection earlier than priming with catL. The varied hydrolytic and cannibalistic interactions between catK and catL when in the presence of a collagen matrix substrate point to the complexities of the

proteolytic network at play in tissue degradation. This study will help to inform potential strategies for proteolytic inhibition to prevent progression of tendon disease and other pathologies characterized by collagen matrix degeneration.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Major Findings

The objective of this dissertation research was to investigate the cooperative role of cathepsins and MMPs in the progression of tendon ECM damage. Previous work has identified cathepsins as major players in rat rotator cuff tendon overuse, osteoporosis and osteoarthritic synovium [5,9,11,59] and shown MMPs upregulated in early stage rat tendon injury [79]. However, collagenolytic enzymes and their endogenous inhibitors are part of a complex proteolytic network with connected and interactive effects on matrix protein hydrolysis. In our previously published data on a rat model of rotator cuff injury, we identified a temporal upregulation of cathepsins K and L in supraspinatus tendon tissue at 4 and 8 weeks of injury, revealing a time-dependent regulatory mechanism leading to tissue damage. To fully characterize this model of injury our studies here extended the time course of injury to 2 and 10 weeks to identify the point of peak upregulation of cathepsins and MMPs. We found evidence of injury in the supraspinatus tendon insertion and degenerative changes in the nearby articular cartilage [80]. However, we observed no upregulation of cathepsins or MMPs at 2 or 10 weeks of injury, suggesting the peak proteolytic upregulation occurred between these two time points. This work revealed the importance of identifying appropriate targets and key points for optimal intervention strategies to prevent the progression of degenerative tendon disorders.

In order to further investigate the cooperative effects of cathepsins on tendon matrix degradation, we developed a collagen gel incubation model and a tendon ECM incubation model. The objective of this study was to identify the protease incubation

sequence that would result in the most matrix substrate degradation. We discovered that in both collagen matrix and type I collagen-rich tendon ECM, initial incubation with cathepsin K primed the matrix for subsequent degradation by cathepsin L. Additionally, we found that soluble and insoluble collagen-rich ECM is able to stabilize active cathepsins K and L and extend proteolysis beyond the expected lifetime of the proteases when not in the presence of substrate. Furthermore, we present evidence that cathepsins K and L exhibit specific distraction and cannibalistic behaviors without substrate and in the presence of a collagen-rich substrate that impact the protease-substrate dynamics such that less matrix degradation occurs. These findings provide key insight into potential consequences of cathepsin behaviors *in vivo*: 1) Prolonged active cathepsins highlight the destructive potential of these proteases even in tissues where only a slight upregulation of an active protease, particularly cathepsin K, occurs. 2) These findings suggest that cannibalism may be a regulatory mechanism to control cathepsin activity extracellularly. 3) This data demonstrates that each of the species in the proteolytic network can impact the other nodes in a way that modulates EC degradation.

Taken together, the work in this dissertation begins to identify some of the complex underlying proteolytic mechanisms that contribute to the degeneration of tendon matrix and surrounding tissues. Furthermore, this work has implications for understanding the degradation of collagen-rich substrates in various pathological contexts. We have identified unique substrate-specific and substrate-independent behaviors of cysteine cathepsins that will be crucial for development of future studies and understanding the functional roles of cathepsins in the greater context of the proteolytic network. The hope is that with the information gathered through this research we are able

to make strides towards developing appropriate and efficacious interventional strategies to prevent tissue degeneration and exploiting robust biological machinery to tune properties of extracellular matrix components *in vivo* and *in vitro* for tissue engineering and enzymology research efforts.

5.2 Limitations and Future Considerations

This work also highlights the need for clinically translational studies to identify appropriate targets and time points of intervention in humans. Because the rat is a quadruped animal, our rat model of rotator cuff injury cannot fully recapitulate human pathology [50]. Still, the data gathered on the behaviors of cathepsin K and cathepsin L may prove crucial to future translational studies and inhibitory strategies. Cathepsin L was upregulated for the longest period of time in our model of rotator cuff injury. However, cathepsin K is a more powerful collagenase. This suggests that both proteases could be viable targets for intervention. Because of how they are temporally regulated, it may be necessary to employ multiple inhibitors at multiple points for maximum efficacy to deter disease progression.

Furthermore, this injury model does not translationally represent the common points of intervention in humans. Typically patients with chronic non-inflammatory tendon injuries have progressed further into the degenerative spectrum before receiving medical intervention than the animals in this research [44,50,51]. This also speaks to the need to develop models to elucidate underlying mechanisms of more acute, inflammatory tendon injuries. Some studies have used rodent models of end stage tendinopathy or full thickness tears and denervation [86], and other studies have analyzed the expression of markers in human torn rotator cuff tendon [5,124], but no animals models have been able

to fully recapitulate early stage inflammatory tendon injury. It is also imperative to find ways to evaluate levels of active proteases in humans that could inform appropriate inhibition strategies. Because we identified that nearby supraspinatus tendon and articular cartilage that share synovial fluid both displayed evidence of damage after injury to the rotator cuff, sampling the level of active enzymes in the synovium may be a promising minimally invasive metric for determining course of action. Identifying evidence of damage to multiple nearby tissues that share synovial fluids highlight the need for additional studies to identify potential pathological changes consistent with full cuff arthropathy [95] to prevent degeneration of multiple tissues in addition to the tendon ECM, such as articular cartilage and rotator cuff muscles [52].

The data in both aims of this dissertation research use rodent models to assess for the amount of active proteases, and thus we focus on the activity of mouse cathepsin L. However, although highly homologous with human cathepsin L, mouse cathepsin is orthologous with human cathepsin V. It has been found that cathepsin V is the most potent mammalian elastase, in addition to displaying some collagenolytic activity [8,15,23]. Further studies are needed to determine if human cathepsin V exhibits the same behaviors—degrading collagen-rich matrix and cannibalistically interacting with catK—as mouse cathepsin L.

Studies have also identified MMP (1, 2, 8, 9, 13, and 14) as potent mammalian collagenases and contribute to the progression of tendinopathy [3,4,21,79]. However, we did not observe upregulation of MMP-2 or MMP-9 at the 2 week or 10 week points of our rat model of tendinopathy [80]. Though we did not capture any upregulation at these time points, this does not mean that MMPs did not contribute to degradation. Further

studies are needed to understand how collagenolytic MMPs contribute to or impede tendon matrix degradation and interact with cathepsins upregulated in this system. Specifically studies are needed to determine how they may enhance, be activated by [68], cannibalize, or serve as a substrate for active cathepsins. MMPs incorporated into the co-incubation experiments with cathepsins and collagen gel matrix or tendon ECM would provide insight into how these families interact in the presence of collagen-rich substrate. Given that catK can activate MMP-9 [68], concurrently incubating MMPs with cathepsins may result in increased cleavage of collagen matrix substrate when compared to matrix that had been pre-incubated with either protease before incubating with the other. These incubations could also answer questions about the impacts of acidic versus neutral microenvironments on the activity of cathepsins and MMPs. MMPs are known to be active at a more neutral pH [7,125], however it has also been hypothesized that local acidification of pericellular space may allow extracellular activity of cathepsins [14].

In all the recombinant cathepsin co-incubations with collagen matrix substrates in Chapter 4, an equimolar amount of each protease was introduced to each reaction. We observed substrate-dependent cannibalistic interactions between cathepsin K and mouse cathepsin L. However, we did not vary the ratio of catK to catL to determine if this impacted the cannibalism dynamics. Because we observed bi-directional cannibalism, by altering the ratio of each protease, one cannibalistic direction may dominate. Varied ratios of catK and catL may also change the patterns of matrix degradation between the concurrent and sequentially incubated groups if an overabundance of one protease prevents binding of the other to the substrate, leaving the unbound protease free to cannibalize or autodigest.

In a tendon injury context, there may not be equimolar amounts of cathepsins or MMPs exposed to the extracellular matrix at a given time. Furthermore, considering that cells are able to continually secrete active proteases, quantifying the amount of active proteases present in early stage tendon injury will also allow for more tunable inhibition strategies. Using zymography, the amount of active cathepsins or MMPs present over a time course of injury in an animal model of tendon injury can be quantified by normalizing densitometric quantification values of band intensities to that of a standard curve with linearly increasing amount run on the same gel. Depending on the targeted proteases and the kinetics of each specific inhibitor, the concentration of inhibitor could then be matched to the protease concentration at the decided point of intervention. These data would be key to determining which inhibition strategy is best, the point of most effective inhibition, and how much inhibitor is necessary to target collagenolytic activity. If developed for translational human studies this could even provide ways of assessing the amount of specific inhibitors needed to prevent collagenolytic activity, even with patient to patient variability.

In addition to incorporating MMPs into the incubation studies with tendon matrix, it may be important to also introduce cathepsin and MMP inhibitors to the incubation reactions as well, to understand how this may interfere with proteolytic degradation of matrix substrate. In an *in vitro* system, it may prove to be difficult to ensure targeted inhibition of only one protease; quantifying the amount of active proteases could be confounding if the inhibitor concentration is too high, impacting the addition of any secondary protease—particularly if the secondary protease is in the same family. Alternatively, if the inhibitor concentration is too low, the primary protease may not be

fully inhibited and still be available to catalytically interact with the substrate or cannibalistically interact with the secondary protease. Beyond recombinant protein experiments, investigating the effects of cathepsin or MMP inhibition on measured amounts of active proteases and tissue degradation in a tendon injury context will determine if there are any counterintuitive cellular responses as a result of inhibition. We know that cathepsins can respond differently to the same inhibitor, and cellular responses to inhibition could be dependent on inhibitor type or inhibitor concentration. These data would be important for successful future animal model or translational studies.

The findings in this dissertation research and the potential future directions highlight an opportunity to use computational modeling as a tool to identify likely interactions of multiple proteases within and between families, and even interactions with their respective inhibitors. Computational tools would provide the ability to predict preferential binding of multiple enzymes and/or inhibitors in the presence of complex and heterogeneous substrates. Instead of experimentally having to determine likely interactions between every species that can drive tendon degradation, we can train models on behaviors of cathepsins, MMPs, and inhibitors on tendon matrix based on data collected in this study and future preliminary work. Data from these studies can identify the most likely interactions and inform experimental approaches to protect tendon matrix from degradation in the presence of multiple proteases.

APPENDIX A: INVESTIGATING THE NECESSITY OF CATHEPSIN K FOR TENDON MATRIX DEGRADATION USING A CATK$^{-/-}$ MOUSE MODEL

A.1. Introduction

Cathepsin K is the most potent mammalian collagenase and plays a large role in tissue remodeling [6,9,11,16,126]. First identified in osteoclasts[6,11,12,16,18], catK is considered to be the primary protease responsible for bone resorption in humans and mice [18,21,22]. Pycnodysostosis is a rare inherited disease that results in a mutation in the catK gene leading to osteopetrotic, dense and brittle bone. People with catK deficiency (catK$^{-/-}$) have dysfunctional osteoclasts that are unable to degrade bone matrix and display overall diminished capacities for tissue remodeling. [6,74] Studies have also found an accumulation of collagen in cellular compartments of fibroblasts from people with pycnodysostosis [75]. A catK$^{-/-}$ mouse model was developed in order to investigate the impact of cathepsin K deficiency on tissue remodeling and potentially develop strategies for preventing osteoporosis [6,74,77].

Due to the reduced capacity for collagen degradation in the catK$^{-/-}$ mouse, this model became advantageous for investigating the necessity of catK for the degradation of tendon matrix. We have shown that tendon primed with catK and then incubated with catL resulted in greater matrix cleavage than concurrent incubations with catK and catL or either protease alone (Fig 4.2). Therefore, we hypothesized that collagen from mice deficient in catK would be less susceptible to cleavage by catL compared to wild type tendons because the deficient tissues would have never been exposed to catK in normal tendon development. We also hypothesized that pre-incubating tendons from catK

deficient and wild type mice, and then incubating with catL would result in similar cleavage observed between the two genotypes.

A.2. Methods

A.2.1. Isolation and homogenization of wild type and cathepsin K deficient mouse Achilles tendons

All animal procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Wild type C57BL/6J mice and mice deficient in cathepsin K were obtained from Jackson Laboratory (Bar Harbor, ME) and raised to 2-4 months of age before being sacrificed. The left and right Achilles tendons were extracted from 12 $catK^{-/-}$ mice and 12 wild type mice as described in section 4.2.2 and frozen at -20°C until needed for homogenization.

Achilles tendons were homogenized in zymography lysis buffer with 0.1mM leupeptin in tissue grinding microcentrifuge tubes with resin (Sigma-Aldrich, St. Louis, MO) and then centrifuged to remove the soluble tendon tissue lysate. One tendon was allocated for each replicate of each condition (n=3/condition) as was described with the individual incubation condition in section 4.2.2. The remaining insoluble tendon ECM was washed in pH6 assay buffer, then centrifuged, and the buffer was discarded in preparation for incubations.

A.2.2. Cathepsin incubations with tendon ECM

Tendon ECM from both wild type and $catK^{-/-}$ mice were incubated with either catL alone or was primed with catK and then subsequently incubated with catL. Cathepsin were incubated at 0.2µM concentrations in pH6 assay buffer with the tendon ECM. All incubations were conducted at 37°C with slight agitation for 8 hrs total. After

the 8hr incubation period, to stop the reaction leupeptin was added to a final concentration of 0.1mM. Samples were centrifuged at 25°C for 15 min and then stored at -20°C until prepped for SDS-PAGE analysis.

A.2.3. SDS-PAGE

In preparation for electrophoresis, each soluble fraction and remaining pellet fraction were prepared with 5X reducing loading dye for SDS-PAGE. 17μL of each sample were loaded into 12.5% polyacrylamide gels and separated by molecular weight. After separation, the polyacrylamide gels were stained with Coomassie Blue and destained, revealing bands indicative of type I collagen α1 and α2 chains, and β-chains, in addition to ECM fragments at various molecular sized throughout the gel.

A.2.4. Statistical Analysis

Statistical significance of densitometric values was determined by a three-way ANOVA in GraphPad Prism between each incubation condition after normalizing to the mean value of the released soluble fragments from the wild type tendon ECM incubated with catL only. Each replicate was repeated at least three times and statistical significance considered when $p<0.05$.

A.3. Results

A.3.1. Varied cleavage and degradation patterns observed between wild type and catK deficient tissues after tendon ECM incubated with catL alone or was primed with catL and then incubated with catL.

To test the hypotheses that incubating $catK^{-/-}$ tendon ECM with catL alone would result in lesser evidence of degradation than wild type tendon ECM, SDS-PAGE was

conducted on samples from both incubation conditions. We observed significantly fewer collagen fragments in the soluble fraction of catK$^{-/-}$ tendon ECM incubated with catL only when normalized to wild type soluble fragments. However, no differences were observed between tissues of each genotype in the tendon pellet fraction when ECM was incubated with catL alone (Fig A.1).

To test the hypothesis that priming catK$^{-/-}$ tendon ECM with catK and then incubating with catL would result in similar amount of cleaved and degraded tendon matrix between catK$^{-/-}$ and wild type tissues, SDS-PAGE was conducted on these samples after the incubation period. No significance was observed between the amount of tendon ECM cleaved in the pellet fraction or degraded in the soluble fraction between the two tissue genotypes, showing that catK and catL were able to hydrolyze substrate from both tissue types. However, much variability was observed between each replicate in all conditions in the pellet fraction and when matrix was incubated first with catK and then catL in the soluble fraction.

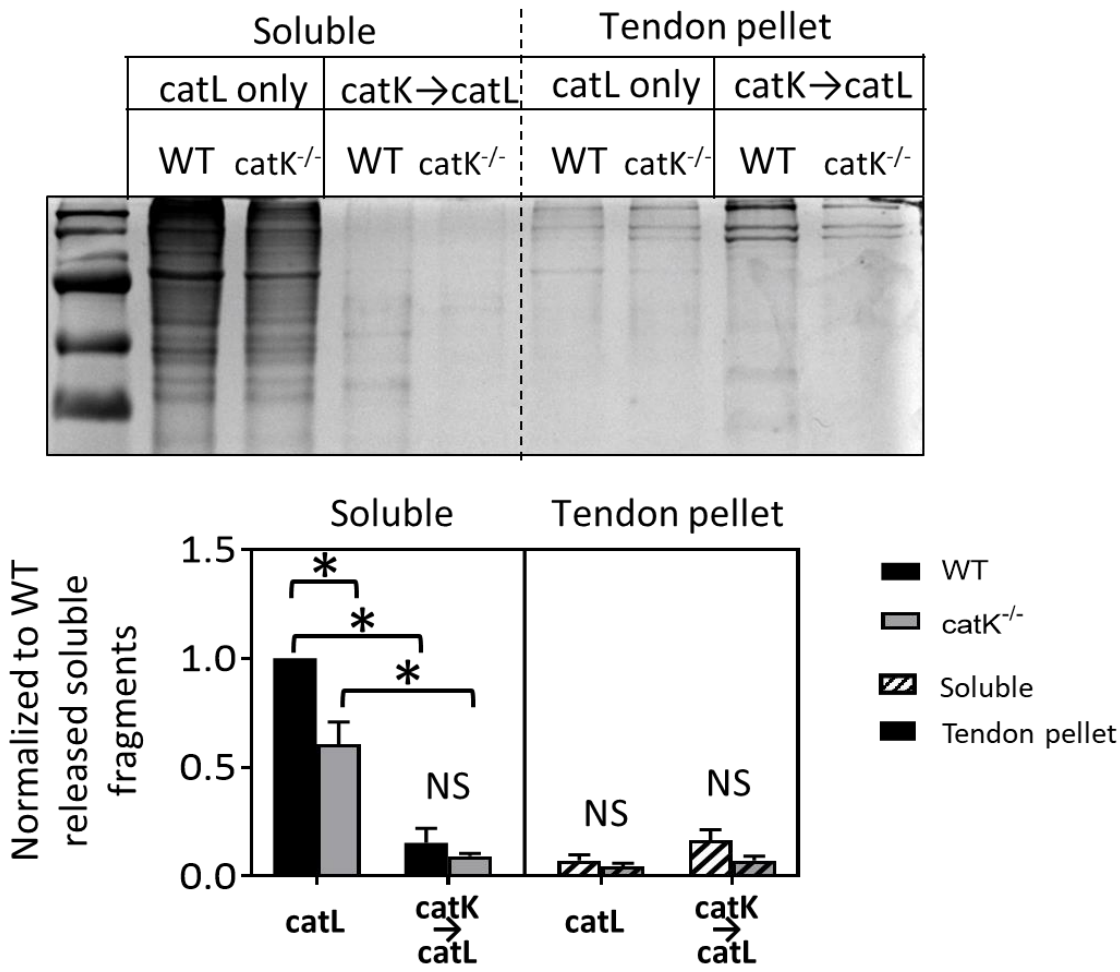

Figure A.1. Varied cleavage and degradation patterns observed between wild type (WT) and catK deficient tissues after tendon ECM incubated with catL alone or was primed with catL and then incubated with catL. CatL alone does not fully degrade soluble tendon ECM fragments, however, significantly fewer fragments were observed with catL incubated with catK$^{-/-}$ tendon ECM than wild type tendon ECM. When primed with catK and then incubated with catL significantly more soluble tendon fragments were degraded in both tissue genotypes than when incubated with catL alone. No differences were measured in the cleaved collagen in the tendon pellet between wild type and catK$^{-/-}$ tissues in either incubation condition.

A.4. Discussion

The objective of this study was to test if exposure to catK *in vivo* was impacted the proteolytic degradation of tendon using tendon ECM from a cathepsin K deficient mouse model. We hypothesized that because catK$^{-/-}$ tendons had never been exposed to catK in tendon development, catL would show diminished degradation capacity when incubated with catK deficient tendon matrix for 8hrs. We also hypothesized that by reintroducing catK in a sequential co-incubation with catL where matrix was first incubated with catK and then incubated with catL, this would result in similar levels of degradation between catK$^{-/-}$ tendon ECM and wild type tendon ECM.

We did observe that, in tissue from mice with either genotype, catL alone was not able to fully degrade soluble tendon fragments, and that significantly more fragments were degraded when ECM was primed catK before incubating with catL. However, the variability between the replicates in the pellet fraction of both incubation conditions and the soluble fraction of the sequential incubation condition made it difficult to draw conclusions from the data. These tendon incubation experiments were conducted using the individual method detailed in section 4.2.2. There, we also observed variability between conditions, leading us to hypothesize that pooling the tendon matrix before incubating with cathepsins would reduce variability. Given the results of this study, it is hypothesized that pooling the tendon ECM before incubating with cathepsins would reduce the confounding variability observed. For that reason, it is recommended to use the tendon ECM pooling method when conducting these specific incubations going forward.

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